

REFERENCE ONLY



2809444033

## UNIVERSITY OF LONDON THESIS

Degree PhD

Year 2007

Name of Author DENISE MARY

O'SULLIVAN

### COPYRIGHT

This is a thesis accepted for a Higher Degree of the University of London. It is an unpublished typescript and the copyright is held by the author. All persons consulting the thesis must read and abide by the Copyright Declaration below.

### COPYRIGHT DECLARATION

I recognise that the copyright of the above-described thesis rests with the author and that no quotation from it or information derived from it may be published without the prior written consent of the author.

### LOAN

Theses may not be lent to individuals, but the University Library may lend a copy to approved libraries within the United Kingdom, for consultation solely on the premises of those libraries. Application should be made to: The Theses Section, University of London Library, Senate House, Malet Street, London WC1E 7HU.

### REPRODUCTION

University of London theses may not be reproduced without explicit written permission from the University of London Library. Enquiries should be addressed to the Theses Section of the Library. Regulations concerning reproduction vary according to the date of acceptance of the thesis and are listed below as guidelines.

- A. Before 1962. Permission granted only upon the prior written consent of the author. (The University Library will provide addresses where possible).
- B. 1962 - 1974. In many cases the author has agreed to permit copying upon completion of a Copyright Declaration.
- C. 1975 - 1988. Most theses may be copied upon completion of a Copyright Declaration.
- D. 1989 onwards. Most theses may be copied.

***This thesis comes within category D.***

☐

This copy has been deposited in the Library of

UCL

☐

This copy has been deposited in the University of London Library, Senate House, Malet Street, London WC1E 7HU.



Evolution of the mechanisms of drug  
resistance in *Mycobacterium tuberculosis*

By

Denise Mary O'Sullivan BSc (Hons)

A thesis submitted to University College London in fulfilment of the  
requirement for the degree of Doctor of Philosophy

Centre for Medical Microbiology  
Department of Infection  
Royal Free & University College Medical School  
Hampstead Campus  
University College London  
Rowland Hill Street  
London NW3 2PF

UMI Number: U594513

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U594513

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.  
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against  
unauthorized copying under Title 17, United States Code.



ProQuest LLC  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106-1346



## **Declaration**

**'I, Denise O' Sullivan, confirm that the work presented in this thesis is my own.**

**Where information has been derived from other sources, I confirm that this has been indicated in the thesis.**

**Signed:**

## Abstract

The aim of this thesis was to investigate the mechanisms by which *Mycobacterium tuberculosis* develops resistance. Resistance depends entirely on genetic alterations within the genome as a consequence of spontaneous mutations.

It is hypothesized that an isoniazid resistant strain, deficient in its response to oxidative damage through a mutation in *katG*, demonstrates a hypermutator phenotype which leads to multiple drug resistance. However there was no evidence of an increased mutation rate. It is likely that compensatory mutations are occurring allowing the bacteria to adapt to its surroundings. The hypothesis that oxidative damage is the major force driving mutations in *M. tuberculosis* was tested by analyzing reported mutations in *rpoB* and *pncA*. The lack of evidence of this type of damage indicates that *M. tuberculosis* is sufficiently competent in repair. This leads to the conclusion that oxidative damage is not the primary mechanism for mutation in the *M. tuberculosis* genome and instead it is the relative fitness of the mutant strain coupled with the resistance phenotype that fixes mutations and permits survival and detection.

Sub-optimal therapy can lead to the selection of mutant strains. It has been shown previously that exposure to sub-inhibitory concentrations of quinolone can increase the mutation rate of mycobacteria. Gene expression profiling was used to determine whether this increase in mutation rate was due to an induction of the SOS repair genes among other DNA repair systems and found that some of these genes were differentially expressed during treatment.

Sub-populations of resistant bacteria may exist in clinical specimens prior to drug exposure and this population may be likely to pre-dominate leading to heterogeneous resistance. This phenomenon called heteroresistance was demonstrated in laboratory strains of *Mycobacterium fortuitum* and *Mycobacterium smegmatis* and in *M. fortuitum* clinical isolates.

## **Acknowledgements**

I would like to acknowledge the support, guidance and help that I received from my supervisors Dr. Tim McHugh and Prof. Stephen Gillespie. In addition to Dr. Tim McHugh for his endless patience, encouragement and inspiration in being a fantastic mentor. I would like to thank the BμG@S group at St. George's, University of London; particularly Prof. Philip Butcher and Dr. Jason Hinds. Thanks to Prof. Stewart Cole, Institute of Pasteur, Paris for provision of the INH34 derived strains.

To all my colleagues and friends at the Royal Free & University College London Medical School for giving me constant laboratory support along with emotional support and kindness; in particular Cassie Pope. Thanks to Anne Dickens for the ordering of materials, Betty Thaine and Fitzroy Hall for autoclaving services.

I am indebted to the support and love that I have received from my family; my mother Catherine for giving me endless words of encouragement, my father Denis, my brother Barry and my aunt Claire. Thanks to all of the friends I have made in London for making my experience in this city a home away from home. To Harry for his support. To my friends in Ireland, particularly Joanne who went through the whole process alongside me.

## LIST OF CONTENTS

<b>Title Page</b>	<b>1</b>
<b>Declaration</b>	<b>2</b>
<b>Abstract</b>	<b>3</b>
<b>Acknowledgements</b>	<b>5</b>
<b>List of Contents</b>	<b>6</b>
<b>List of Tables</b>	<b>13</b>
<b>List of Figures</b>	<b>15</b>
<b>Abbreviations</b>	<b>17</b>

## CHAPTER 1: INTRODUCTION

1.1 History	19
1.2 Classification of Mycobacteria	22
1.2.1 The <i>Mycobacterium</i> genus	
1.2.2 The <i>M. avium-intracellulare</i> complex	
1.2.3 Other slow growing mycobacteria	
1.2.4 Rapid growers	
1.3 Morphology	27
1.4 The <i>M. tuberculosis</i> Genome	29
1.5 Mycobacterial Cell Wall	33
1.5.1 Cell wall composition	
1.5.2 The cord factor	
1.5.3 Characteristics of the mycobacterial cell wall	
1.6 Epidemiology	36
1.6.1 Spread of disease	
1.6.2 Global burden	
1.6.3 A tuberculosis epidemic	
1.6.4 Case detection	
1.6.5 Risk population	
1.6.6 The impact of immigration	
1.6.7 Risk factors leading to disease	
1.6.8 Impact of Directly Observed Therapy – Short Course	
1.6.9 Epidemiological molecular tools	
1.7 Pathogenicity	47
1.7.1 Development of disease	
1.7.2 Latency and reactivation	
1.7.2.1 Development of latent and reactivation tuberculosis	
1.7.2.2 Location of persistent bacilli	

1.8 Clinical Presentation of Tuberculosis	54
1.9 Diagnosis of Tuberculosis	56
1.9.1 Detection of <i>M. tuberculosis</i> in clinical isolates	
1.9.2 Liquid culture methods	
1.9.3 Tuberculin skin test	
1.9.4 Molecular detection of <i>M. tuberculosis</i>	
1.10 Treatment	61
1.10.1 The population theory	
1.10.2 Treatment regimens	
1.11 Drug Resistance	66
1.11.1 Rifampicin	
1.11.1.1 Activity of rifampicin	
1.11.1.2 Rifampicin resistance	
1.11.1.3 Rifampicin derivatives	
1.11.2 Isoniazid	
1.11.2.1.a Activity of isoniazid	
1.11.2.1.b Catalase-peroxidase activity	
1.11.2.2 Isoniazid resistance	
1.11.2.2.a <i>katG</i>	
1.11.2.2.b <i>inhA</i>	
1.11.2.2.c <i>kasA</i>	
1.11.2.2.d <i>ahpC</i>	
1.11.3 Pyrazinamide	
1.11.3.1 Activity of pyrazinamide	
1.11.3.2 Pyrazinamide resistance	
1.11.4 Fluoroquinolones	
1.11.4.1 Generations of fluoroquinolone	
1.11.4.2 DNA gyrase	
1.11.4.3 Development of quinolone resistance	
1.11.4.4 Quinolone resistance due to <i>gyrA</i> and <i>gyrB</i> mutations	
1.11.4.5 Quinolone resistance due to other mechanisms	
1.11.4.6 Induction of the SOS response by fluoroquinolones	
1.11.5 Streptomycin	
1.11.6 Ethambutol	
1.12 Heteroresistance	96
1.12.1 The Study of Heteroresistance	
1.12.2 Causes of Heteroresistance in Mycobacteria	
1.12.2.1 Antibiotic Penetrating Ability	
1.12.2.2 Inadequate Therapy	
1.12.2.3 Growth Cycle of Bacteria	
1.12.2.4 Defective DNA Repair System	
1.12.3 Detection of Heteroresistance	
1.12.4 Clinical Significance	
1.12.5 Genetic Mechanism of Heteroresistance	

1.13 Multi-drug Resistant Tuberculosis	104
1.13.1 Epidemiology of MDR-TB	
1.13.2 Treatment of MDR-TB	
1.13.3 Genetic mechanisms of multiple drug resistance	
1.14 Mutation	110
1.14.1 Mutagens	
1.14.2 Oxidative Stress	
1.14.2.1 Causes of oxidative stress	
1.14.2.2 Protective mechanisms against reactive oxygen intermediates	
1.14.2.3 Oxidative damage to DNA	
1.14.2.4 The GO Repair System	
1.14.2.5 Other mutagenic effects on DNA	
1.14.3 Mechanism of mutation repair in <i>M. tuberculosis</i>	
1.14.3.1 The SOS response	
1.14.3.2 Induction of the SOS response	
1.14.3.3 Differences in Gene Expression between <i>E. coli</i> and <i>M. tuberculosis</i> following DNA damage	
1.14.3.4 Binding of <i>lexA</i> and <i>recA</i>	
1.14.3.5 Other repair systems	
1.14.3.5.a Recombinational repair	
1.14.3.5.b Base excision repair	
1.14.3.5.c Nucleotide excision repair	
1.14.3.5.d Mismatch repair	
1.14.4 Mutation Rates	
1.14.4.1 Determining mutation rates	
1.14.4.2 Design of mutation rate experiments	
1.14.4.3 Assumptions of fluctuation analysis	
1.15 Fitness	134
1.16 Aims of thesis	137

## CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Preparation of Culture	138
2.1.1 Middlebrook 7H9 broth	
2.1.1a Albumin Dextrose Enrichment	
2.1.2 Middlebrook 7H10 agar	
2.1.3 Mueller Hinton broth	
2.1.4 Mueller Hinton agar	
2.1.5 Blood Agar	
2.2 Preparation of Buffers and Solutions	140
2.2.1 0.5M EDTA	
2.2.2 1M Tris	
2.2.3 5M NaCl	



2.2.4 TE Buffer	
2.2.5 10% SDS	
2.2.6 CTAB/NaCl	
2.2.7 Lysozyme	
2.2.8 Proteinase K	
2.2.9 SDS/Proteinase K	
2.2.10 Tris-Borate EDTA buffer (TBE)	
2.2.11 5M Guanidine thiocyanate (GTC) solution	
2.3 Miles and Misra Plate Count	143
2.4 Minimum inhibitory concentration (MIC) determination	144
2.4.1 Determining of the MIC of moxifloxacin to <i>M. fortuitum</i> NCTC 10394	
2.4.2 Determination of MIC's using the multi-inoculator system	
2.4.3 Isolation of resistant daughter colonies and subsequent MIC determination	
2.5 Mutation rate determination using the $p_o$ method	148
2.5.1 Mutation rate determination following exposure to hydrogen peroxide	
2.5.2 Screening of rifampicin resistant colonies for mutations within <i>rpoB</i>	
2.6 Determination of mutation frequency after exposure to oxidative stress	151
2.7 Nucleic acid extraction and clean-up	152
2.7.1 Isolation of genomic DNA	
2.7.2 Crude DNA isolation	
2.7.3 RNA extraction	
2.7.4 RNA cleanup	
2.8 Polymerase chain reaction	157
2.8.1 Amplification of the gyrase A ( <i>gyrA</i> ) subunit of <i>M. fortuitum</i>	
2.8.2 Amplification of the RRDR of rifampicin resistant colonies	
2.8.3 Agarose Gels	
2.8.4 RNA quantification	
2.8.4.1 Bioanalyser system	
2.8.4.2 Agarose gel electrophoresis	
2.8.5 Gel Photography	
2.8.6 Sequencing of samples	
2.8.7 Quantitative Reverse Transcriptase Real time PCR (qRT-PCR) Procedure	
2.9 B $\mu$ G@S Microarray Protocol	164
2.9.1 RNA versus DNA: Labelling and Hybridisation	
2.9.2 Cy5 label DNA	
2.9.3 Cy3 label RNA	
2.9.4 Prehybridise slide	
2.9.5 Qiagen MinElute Purification to purify Cy3/Cy5 labelled cDNA/DNA	

- 2.9.6 Wash Preparation
- 2.9.7 Hybridise Slide
- 2.9.8 Wash Slide
- 2.9.9 Scan Slide
- 2.9.10 Data Analysis

## CHAPTER 3: HETERORESISTANCE

3.1 Introduction	172
3.1.1 <i>M. fortuitum</i> as a Model System of <i>M. tuberculosis</i>	
3.1.2 Aim of chapter	
3.2 Materials and Method	173
3.3 Results	173
3.3.1 Experimental Assumptions	
3.3.2 MIC Distribution of Control Strains	
3.3.3 MIC Distribution of Clinical Isolates	
3.3.3.1 Experimental Replicates	
3.3.3.2 MIC's of <i>M. fortuitum</i> RF 01:332	
3.3.3.3 MIC's of <i>M. fortuitum</i> RF 02:159 and RF 02:173	
3.3.4 Reversion of Resistance	
3.3.5 Screening of the QRDR of <i>gyrA</i> of resistant colonies	
3.4 Discussion	182
3.4.1 Experimental Findings	
3.4.2 Heteroresistance in <i>M. fortuitum</i> Clinical Isolates	
3.4.3 Heteroresistance in Laboratory Strains	
3.4.4 Sub-population of Resistant Bacteria	
3.4.5 Reversion of Resistant Colonies	
3.4.6 Genetic Determinants of Heteroresistance	
3.4.7 Heteroresistance is not restricted to a particular drug	
3.4.8 Clinical Significance of Heteroresistance	
3.4.9 Experimental limitations and future work	
3.5 Conclusion	189

## CHAPTER 4: AN INSIGHT INTO THE ROLE OF OXIDATIVE STRESS IN *M. TUBERCULOSIS* EVOLUTION

4.1 Introduction	190
4.1.1 Aim of this chapter	
4.2 Materials and Method	191
4.3 Results	192

4.3.1 Geographical location of isolates analysed	
4.3.2 Exclusions from study	
4.3.3 Mutation detection	
4.3.4 <i>rpoB</i> mutations	
4.3.5 <i>pncA</i> mutations	
<b>4.4 Discussion</b>	<b>200</b>
4.4.1 Hypothesis	
4.4.2 G·C → A·T transitions	
4.4.3 Transversions	
4.4.4 <i>pncA</i> mutations	
4.4.5 Limitations of the study	
4.4.6 Fitness determines the distribution of <i>rpoB</i> mutations	
4.4.7 Absence of mismatch repair	
<b>4.5 Conclusion</b>	<b>207</b>
<b>CHAPTER 5: PROTECTION AGAINST OXIDATIVE DAMAGE</b>	
<b>5.1 Introduction</b>	<b>208</b>
5.1.1 Aim of Chapter	
<b>5.2 Materials and Method</b>	<b>209</b>
5.2.1 Strain Characteristics	
5.2.2 Determining the requirement of the strains for catalase	
5.2.3 Determination of mutation rate	
5.2.4 Screening of rifampicin resistant colonies for mutations within <i>rpoB</i>	
<b>5.3 Results</b>	<b>212</b>
5.3.1 Pure growth of strains on media	
5.3.2 Growth of strains without catalase	
5.3.3 Mutation rates	
5.3.4 Mutation frequency of INH34 <sup>pAP01</sup> , INH34 <sup>pAP23</sup> and INH34 <sup>pPD28</sup>	
5.3.5 Sequencing of <i>rpoB</i>	
<b>5.4 Discussion</b>	<b>216</b>
5.4.1 Proposed Hypothesis	
5.4.2 Estimating Mutation Rates using the $p_0$ Method	
5.4.3 INH34 Transformed Strains with Differing <i>katG</i> Alleles	
5.4.4 Mutation Rate Results	
5.4.5 Presence of Exogenous Catalase	
5.4.6 Compensation by <i>ahpC</i> for Deficiency of Catalase-Peroxidase Activity	
5.4.7 Pattern of Mutations in <i>rpoB</i>	
<b>5.5 Conclusion</b>	<b>223</b>

## **CHAPTER 6: THE EFFECT OF SUB-INHIBITORY CONCENTRATIONS OF CIPROFLOXACIN ON THE DNA REPAIR RESPONSES OF *M. TUBERCULOSIS***

### **6.1 Introduction 224**

#### 6.1.1 The Use of DNA Microarrays

#### 6.1.2 Aim

### **6.2 Materials and Method 227**

#### 6.2.1 Quinolone treatment of culture

#### 6.2.2 Bioassay: to check the activity of the batch of ciprofloxacin

### **6.3 Results 229**

### **6.4 Discussion 240**

#### 6.4.1 Gene expression pattern

#### 6.4.2 Regulation of DNA repair genes

#### 6.4.3 Genes identified from other expression profiling studies

#### 6.4.4 The effect of sub-inhibitory concentrations of antibiotics on gene expression

#### 6.4.5 General cellular responses

#### 6.4.6 Data analysis

### **6.5 Conclusion 253**

## **CHAPTER 7: FINAL DISCUSSION 254**

## **REFERENCES 263**

## **APPENDIX: Published Papers 339**

Analysis of *rpoB* and *pncA* mutations in the published literature: an insight into the role of oxidative stress in *Mycobacterium tuberculosis* evolution?

O'Sullivan DM, McHugh TD, Gillespie SH. 2005. Journal of Antimicrobial Chemotherapy **55**: 674-679.

Effect of subinhibitory concentrations of ciprofloxacin on *Mycobacterium fortuitum* mutation rates. Gillespie SH, Basu S, Dickens AL, O' Sullivan DM, McHugh TD. 2005. Journal of Antimicrobial Chemotherapy **56**: 344-348.

## LIST OF TABLES

### CHAPTER 1

Table 1.1:	Recommended dosages of standard antituberculosis drugs.	65
Table 1.2:	Effect on the mutation rate of <i>M. fortuitum</i> (RF 01:332) when grown in the presence of sub-inhibitory levels of ciprofloxacin	88
Table 1.3:	The effect of mutagens on bacteria.	111
Table 1.4:	Parameters for the methods used in determining mutation rates	129

### CHAPTER 2

Table 2.1:	Experimental replicates for each strain and drug	146
Table 2.2:	Primer sequences designed to amplify segments of <i>rpoB</i>	159
Table 2.3:	Probes and primers used for qRT-PCR	163

### CHAPTER 3

Table 3.1:	The variation in the determined MIC values for each isolate to moxifloxacin, minocycline and amikacin.	178
Table 3.2:	The proportion of colonies that are resistant to each drug ( $\geq 2 \times$ MIC) for each strain and which strains were heteroresistant as determined by each drug.	179
Table 3.3:	Results for clonal population 12, a first generation moxifloxacin mutant of <i>M. fortuitum</i> RF 01:332 which had a MIC of $> 2 \mu\text{g/mL}$ moxifloxacin and $0.7 \mu\text{g/mL}$ minocycline which was then re-tested.	179

### CHAPTER 4

Table 4.1:	The geographical location of <i>M. tuberculosis</i> isolates analysed for mutations in <i>rpoB</i>	192
Table 4.2:	The geographical location of <i>M. tuberculosis</i> isolates analysed for mutations in <i>pncA</i>	193
Table 4.3:	The distributions of mutations within the <i>rpoB</i> gene of 840 clinical isolates of <i>M. tuberculosis</i> .	196
Table 4.4:	The distributions of mutations within the <i>pncA</i> gene of 114 clinical isolates of <i>M. tuberculosis</i> .	198

### CHAPTER 5

Table 5.1:	Strain genotype and corresponding enzymatic activity	209
Table 5.2:	Mutation rate for each strain	213
Table 5.3:	Summary of strains and mutation rates	214
Table 5.4:	Colony counts from 3 replicate experiments before the catalase had been removed from the media.	214

**CHAPTER 6**

Table 6.1:	Experiments Performed	227
Table 6.2:	Results of a database search for genes involved in DNA repair, mutagenesis and recombination in <i>M. tuberculosis</i> which were further studied in the experiment.	229
Table 6.3:	Colony counts of bacteria comparing the number of bacteria recovered from each treatment demonstrating no reduction in bacterial numbers	230
Table 6.4:	The inhibition of <i>E. coli</i> ATCC 25922 using different dilutions of ciprofloxacin.	231
Table 6.5:	Number of genes with statistically significant differences analysed using ImaGene™ and Bluefuse™ software.	232
Table 6.6.a-d:	Down- and up- regulated genes from DNA repair, recombination and mutagenesis gene list (110 genes) with statistically significant differences among Time, MIC for 4hr and 12hr treatments with ½ and ¼ MIC ciprofloxacin	233
Table 6.6.a:	Gene expression 4hr treated with ½ MIC ciprofloxacin normalised to time 0hr untreated control.	233
Table 6.6.b:	Gene expression 4hr treated with ¼ MIC ciprofloxacin normalised to time 0hr untreated control.	234
Table 6.6.c:	Gene expression 12hr treated with ½ MIC ciprofloxacin normalised to time 0hr untreated control.	235
Table 6.6.d:	Gene expression 12hr treated with ¼ MIC ciprofloxacin normalised to time 0hr untreated control.	235
Table 6.7:	The response of <i>M. tuberculosis</i> after 4hr and 12hr treatment with ½ and ¼ MIC ciprofloxacin examined by functional category, as defined by Cole et al 1998.	237
Table 6.8:	The response of <i>M. tuberculosis</i> after 4hr and 12hr treatment with ½ and ¼ MIC ciprofloxacin classified by pathway.	238
Table 6.9:	The mean relative fold change and the standard error of the mean (S.E.M.) of 2 biological replicates, calculated by the equation $2^{-\Delta\Delta CT}$ , demonstrating the expression of <i>dnaE2</i> normalised to <i>sigA</i> after treatment with ¼ and ½ MIC ciprofloxacin over time.	239

## LIST OF FIGURES

### CHAPTER 1

Figure 1.1:	Phylogenetic tree of rapidly and slowly growing mycobacteria	26
Figure 1.2:	Thin section transmission electron micrograph of <i>M. tuberculosis</i>	28
Figure 1.3:	The genome of <i>M. tuberculosis</i> H37Rv (Cole et al 1998)	32
Figure 1.4:	Cell wall of <i>M. tuberculosis</i>	35
Figure 1.5:	Pathogenesis of disease by <i>M. tuberculosis</i>	50
Figure 1.6:	Hypothetical picture of sub-populations of tubercle bacilli within lesions	62
Figure 1.7:	Frequency distribution of resistance mutations in <i>rpoB</i> of clinical isolates of <i>M. tuberculosis</i> occurring in codons 507-533.	71
Figure 1.8:	The structures of the generations of quinolone development	84
Figure 1.9:	Oxidative Damage can lead to GO lesions in DNA	117
Figure 1.10:	The SOS response in <i>E. coli</i>	121

### CHAPTER 2

Figure 2.1:	Schematic representation of the method for determining clonal population MIC's within a broth culture of an isolate.	147
-------------	--	-----

### CHAPTER 3

Figure 3.1:	An illustrative example of a strain with normal MIC pattern, <i>M. fortuitum</i> RF 02:159, and a strain with a heteroresistant pattern, <i>M. fortuitum</i> RF 01:332	180
Figure 3.2:	Sequencing of the QRDR of <i>M. fortuitum gyrA</i>	181

### CHAPTER 4

Figure 4.1:	The distribution of mutations within the <i>rpoB</i> gene (accession no. Rv0667) in <i>M. tuberculosis</i> .	197
Figure 4.2:	The distribution of mutations within the <i>pncA</i> gene (accession no. Rv2043c) in <i>M. tuberculosis</i> .	199

### CHAPTER 5

Figure 5.1:	Construction of plasmids to complement the $\Delta furA$ - <i>katG</i> strain of <i>M. tuberculosis</i> with different <i>katG</i> alleles to generate pAP21, pAP22 and pAP23.	210
Figure 5.2:	Alignment of <i>rpoB</i> sequences showing the mutations detected.	215



**CHAPTER 6**

- Figure 6.1: Venn diagram showing the overlap of genes identified by the Benjamini and Hochberg False Discovery Rate Analysis comparing the different treatments at the 4hr time point. **236**
- Figure 6.2: Venn diagram showing the overlap of genes identified by the Benjamini and Hochberg False Discovery Rate Analysis comparing the different treatments at the 12hr time point. **236**

## Abbreviations

5-OH-C:	5-hydroxycytosine
5-OH-U:	5-hydroxyuracil
8-oxodA:	8-oxodeoxyadenine
8-oxodG:	7,8-dihydro-8-oxodeoxyguanine
A:	adenine
ACP:	acyl carrier protein
ADC:	albumin dextrose catalase
ANOVA:	analysis of variance between groups
ATPase:	Adenosine triphosphatase
BAC:	Bacterial Artificial Chromosome
BCG:	Bacile Calmette-Guérin
C:	cytosine
C:	number of parallel cultures
cfu/mL:	colony forming units/mL
Cg:	5,6-dihydrocytosine
CR:	Complement Class Receptors
DIM:	phthiocerol dimycocerosate
DLT:	dominant lethal test
DNA:	deoxyribonucleic acid
DOTS:	Directly Observed Therapy – Short Course
EBA:	early bactericidal activity
ELISA:	enzyme linked immunosorbent assay
ELISPOT:	enzyme linked immunoSpot Assay
FAS:	fatty acid synthetase
G:	guanine
GM-CSF:	granulocyte-macrophage colony-stimulating factor
GTPase:	guanosine triphosphatase
H <sub>2</sub> O <sub>2</sub> :	hydrogen peroxide
HPLC:	high performance liquid chromatography
IFN:	interferon
IL:	interleukin
IS:	insertion sequence
LAM:	lipoarabinomannan
LJ Medium:	Löwenstein-Jensen medium
LM:	lipomannan
m:	number of mutations
MAC:	<i>Mycobacterium avium-intracellulare</i> complex
mAGP:	mycolyl arabinogalactan-peptidoglycan
MAVI:	modular algorithms for volume images
MCP-1:	monocyte chemoattractant protein 1
MDR-TB:	Multi-drug resistant tuberculosis
MHC:	Major histocompatibility complex
MIC:	minimum inhibitory concentration
MIRU:	mycobacterial interspersed repetitive units
MMC:	mitomycin C
MNNG:	N-methyl-N'-nitro-N-nitrosoguanidine
MNT:	photo-micronucleus test
MOTT:	Mycobacteria other than those of the <i>M. tuberculosis</i> complex

MPTR:	major polymorphic tandem repeat
MRSA:	methicillin resistant <i>Staphylococcus aureus</i>
NAAT:	nucleic acid amplification techniques
NAD:	Nicotinamide adenine dinucleotide
NADH:	The reduced form of NAD
NCTC:	National Collection of Type Cultures
NK cells:	natural killer
N <sub>0</sub> :	initial inoculum
NOS:	nitric oxide synthase
NRP:	non-replicating persistence
N <sub>f</sub> :	final inoculum
OADC:	oleic acid albumin dextrose catalase
PCR:	polymerase chain reaction
PGRS:	polymorphic guanine cytosine-rich repetitive sequence
PIMs:	the phosphatidylinositol mannosides
p <sub>0</sub> :	number of cultures with no mutants
PPD:	purified protein derivative
QRDR:	quinolone resistance determining region
qRT-PCR:	quantitative reverse transcriptase real time PCR
RANTES:	Regulation-upon-activation, normal T expressed and secreted
RFLP:	restriction fragment length polymorphism
RNA:	ribonucleic acid
RNI:	reactive nitrogen intermediates
ROI:	reactive oxygen intermediates
RRDR:	rifampicin resistance determining region
SDA:	strand displacement amplification
SDW:	sterile distilled water
SEM:	standard error of the mean
SSCP:	single strand conformation polymorphism
T:	thymine
TACO:	tryptophan-aspartate containing coat protein
TLR:	toll like receptor
TNF- $\alpha$ :	tumour necrosis factor alpha
Ug:	5,6-dihydroxy-5,6-dihydrouracil
VNTR:	variable numbers of tandem repeat
WHO:	World Health Organisation
ZN Stain:	Ziehl-Neelsen stain
$\mu$ :	mutation rate

## Chapter 1: Introduction

### 1.1 History of Tuberculosis

Tuberculosis is a chronic granulomatous disease caused by infection with some species of mycobacteria. *Mycobacterium tuberculosis* is a pathogen which has been present in the human population throughout recorded history: the earliest evidence is from a female skeleton from 5800 B.C. (Canci, Minozzi, & Borgognini Tarli 1996), signs of tubercular decay have been traced in the spinal columns of Egyptian mummies from 4500 B.C. (Zias 1998). The term phthisis, a Greek word meaning consumption 'I am wasting', appears in literature around 460 B.C. when Hippocrates identified the disease as being widespread among the Greek population. The early cases of tuberculosis were probably sporadic, but as population densities began to increase then the disease began to spread more widely (Daniel, Bates, & Downes 1994).

In the early seventeenth century people began to describe the exact pathological and anatomical distributions of the disease. By the mid 1600s The London Bills of Mortality recorded that tuberculosis was the causative agent of 20% of deaths in England (Reichler et al. 2002). There is evidence in Medieval Europe of the disease from several sources which showed that the incidence of tuberculosis seemed to rise with population density. In his 'Opera Medica' written in 1679, Sylvius described actual tubercles in the lungs and other areas of consumptive patients. Fracastorius (1483-1553) suggested in his 'De Contagione' that tuberculosis was due to germs carrying the disease. In the seventeenth and eighteenth centuries, the "White Plague"

as TB was commonly referred to, had a mortality rate of 1 in 5 adults (Iseman 1994). During the industrial revolution, the distribution of human population changed from agrarian to urban communities which brought overcrowding, poor sanitation and malnutrition. This made the population more susceptible to *M. tuberculosis* which was spreading as a result (Cole 2002; Daniel, Bates, & Downes 1994). The first of many sanatoria opened in Germany in 1859 which isolated the sick while providing adequate nutrition.

In the late 19<sup>th</sup> century, it was also discovered that the disease was not random but was caused by a specific microorganism. Then in 1882, Robert Koch developed a staining technique which identified the tubercle bacillus (Koch 1882). He demonstrated that this slow-growing mycobacterium was the agent of human disease using a guinea pig model. In 1895, Wilhem Conrad von Röntgen discovered the x-ray which could view the progress and severity of a patient's disease (Greene 1992). By 1897, the theory that TB was transmitted by aerosol droplets was established (Meachen 1936).

Another important development was the discovery of Calmette and Guérin, who used a virulent *Mycobacterium bovis* isolate and attenuated the isolate by 230 serial passages in a glycerol, potato-extract and bile salts broth (Calmette 1927). This is the origin of the BCG (bacille Calmette-Guérin) vaccine which is used widely today as a live vaccine which protects humans against tuberculosis and leprosy (Karonga Prevention Trial Group 1996). The attenuation of the BCG is probably as a result of a serial loss of genetic material and has been shown with strong testimony to be safe

with negligible side effects after the administration of nearly 3 billion doses (Bloom & Fine 1994).

From 1850-1950 it is estimated that tuberculosis killed one billion persons (Iseman 1994). As the standard of living improved in Western Europe and in the United States throughout the late nineteenth and twentieth centuries, the incidence of TB declined. This was attributed to the improvement in living conditions and the decline of poverty. By 1985, the species of *M. tuberculosis* was defined by DNA/DNA hybridisation studies (Imaeda 1985) and was subsequently characterised by Sreevatsan et al by its lack of diversity in the bulk of its genes (Sreevatsan et al. 1997a).

## 1.2 Classification of Mycobacteria

### 1.2.1 The *Mycobacterium* Genus

Mycobacteria belong to the order Actinomycetales, family Mycobacteriaceae and the genus *Mycobacterium*. There is a close genetic relationship between the genera *Mycobacterium*, *Corynebacterium*, *Actinomyces* and *Nocardia* which all appear as Gram-positive irregular non-sporing rods. The genus *Mycobacterium*, meaning fungus-bacterium, contains more than 85 described species (figure 1.1). The majority of mycobacteria are ubiquitous environmental organisms which inhabit the soil and rarely cause disease in humans (Holland 2001). Forty two new mycobacterial species have been officially recognised in the last 15 years (Tortoli 2003). Only a minority of species are obligate human pathogens which include the organisms of the *M. tuberculosis* complex. Organisms in this complex have similar growth characteristics and probably arose as an ecological niche change which resulted in increased pathogenicity in mammals. The members of the complex share >99% identity at the nucleotide level but they differ in morphology, host range, disease patterns in experimental models and have small biochemical differences (Brosch et al. 2002). The complex is comprised of six members: *M. tuberculosis*, the causative agent in the majority of human tuberculosis cases; a sub-Saharan variant *Mycobacterium africanum*; the closely related *M. bovis* which infects in particular cattle and badgers among other mammals; the attenuated variant *M. bovis* BCG; *Mycobacterium microti* which causes tuberculosis in voles and wood mice but is avirulent in humans; and *Mycobacterium canettii*, which rarely infects humans (Cole 2002). The pathology and course of *M. tuberculosis*, *M. bovis* and *M. africanum* disease in humans are similar.



It was thought that *M. tuberculosis* is the ancestor of *M. bovis* as tuberculosis was acquired from cattle after the domestication of livestock which led to a more agricultural lifestyle (Haas & Haas 1996). However recent genomic analysis involving the mapping of the distribution of a series of DNA deletions among closely related mycobacteria confirms that *M. tuberculosis* is the ancestral species (Brosch et al. 2002; Mostowy et al. 2002).

*Mycobacterium leprae*, the causative agent of leprosy, has traditionally been classed as a member of the genus *Mycobacterium*. However some authors have suggested that it should be classified with members of the genera *Nocardia* and *Rhodococcus* as it has some different properties to other mycobacteria such as the cell wall component glycine instead of lysine which is found in other mycobacteria. *M. leprae* cannot be grown *in vitro* (Hastings 1994) and must be grown in either the nine banded armadillo, a natural host or in the nude mouse footpad. *M. tuberculosis* infection and BCG vaccination may protect against leprosy (Karonga Prevention Trial Group 1996).

### 1.2.2 The *M. avium-intracellulare* Complex

Other species which are members of the environmental mycobacteria group but have the potential to be pathogenic in humans include those in the *M. avium-intracellulare* complex (MAC), which is comprised of the species *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* (Selkon 1969). *Mycobacterium avium* subsp. *paratuberculosis*, a significant pathogen of livestock (Johne's Disease) and implicated in causing Crohn's disease in humans, is also a member of MAC (Pickup et al. 2005). Approximately 10% of pulmonary infection

resulting from mycobacteria is caused by MAC in south-eastern USA, Japan and Western Australia (Edwards 1970; Selkon 1969). Those with MAC disease are often immunocompromised. MAC bacilli are extremely widespread in nature and are common in the soil environment and in surface waters. There is little evidence to implicate that transmission occurs between hosts but exposure to the MAC bacilli is not uncommon from tap water. MAC infection is notoriously difficult to treat due to intrinsic drug resistance. The organisms that make up this complex show remarkable genetic plasticity.

### 1.2.3 Other Slow Growing Mycobacteria

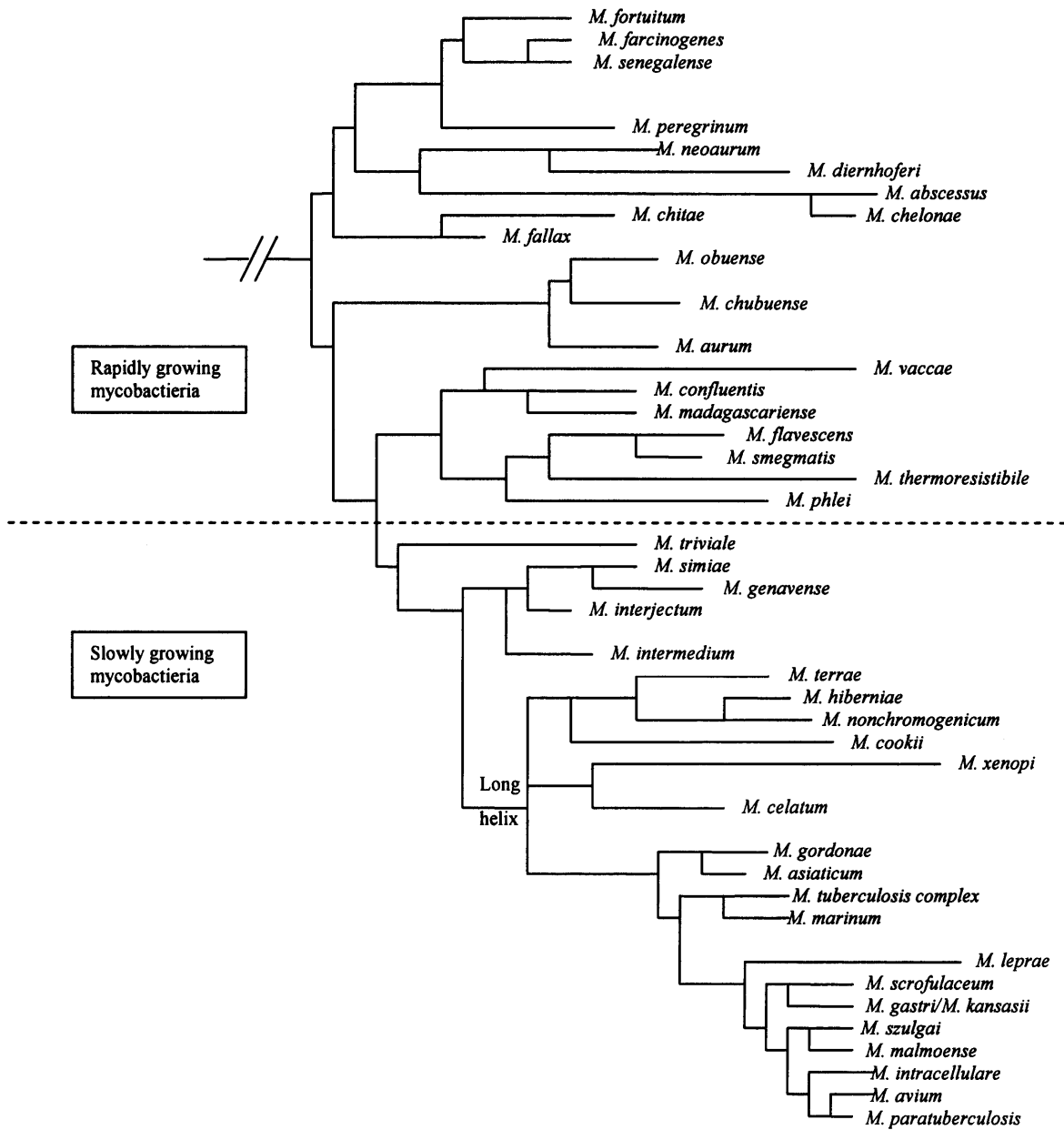
A close relative of *M. avium-intracellulare* is *Mycobacterium malmoeense* which unlike MAC has no known environmental reservoir. *Mycobacterium kansasii* and *Mycobacterium xenopi* are responsible for chronic pulmonary infection. *M. kansasii* infection has been associated with the HIV epidemic in North America and Europe (Selkon 1969). The slow growing *Mycobacterium ulcerans*, the causative agent of Buruli ulcer is probably one of the only pathogenic *Mycobacterium* species which does not have a significant intracellular existence (George et al. 1999). The generation time of *M. ulcerans* and members of the *M. tuberculosis* complex is 20hr. *M. ulcerans* is an evolutionary derivative of *Mycobacterium marinum* (section 1.2.4).

### 1.2.4 Rapid Growers

The group of rapid growers include the environmental saprophytes *Mycobacterium fortuitum* and *Mycobacterium chelonae* which are phylogenetically older than the slow

growers (Pitulle et al. 1992). They may also be found in human specimens without causing disease and are principally skin and soft tissue pathogens. *M. marinum* infects the skin, usually through water contact. *M. marinum* is emerging as a model for *M. tuberculosis* pathogenesis as it is one of the closest relatives of the *M. tuberculosis* complex organisms. *M. marinum* grows rapidly at a generation time of 4hr at 33°C. *Mycobacterium smegmatis* is a saprophytic species of mycobacteria which rarely cause human disease. Environmental mycobacteria are also commonly referred to as mycobacteria other than those of the *M. tuberculosis* complex (MOTT).

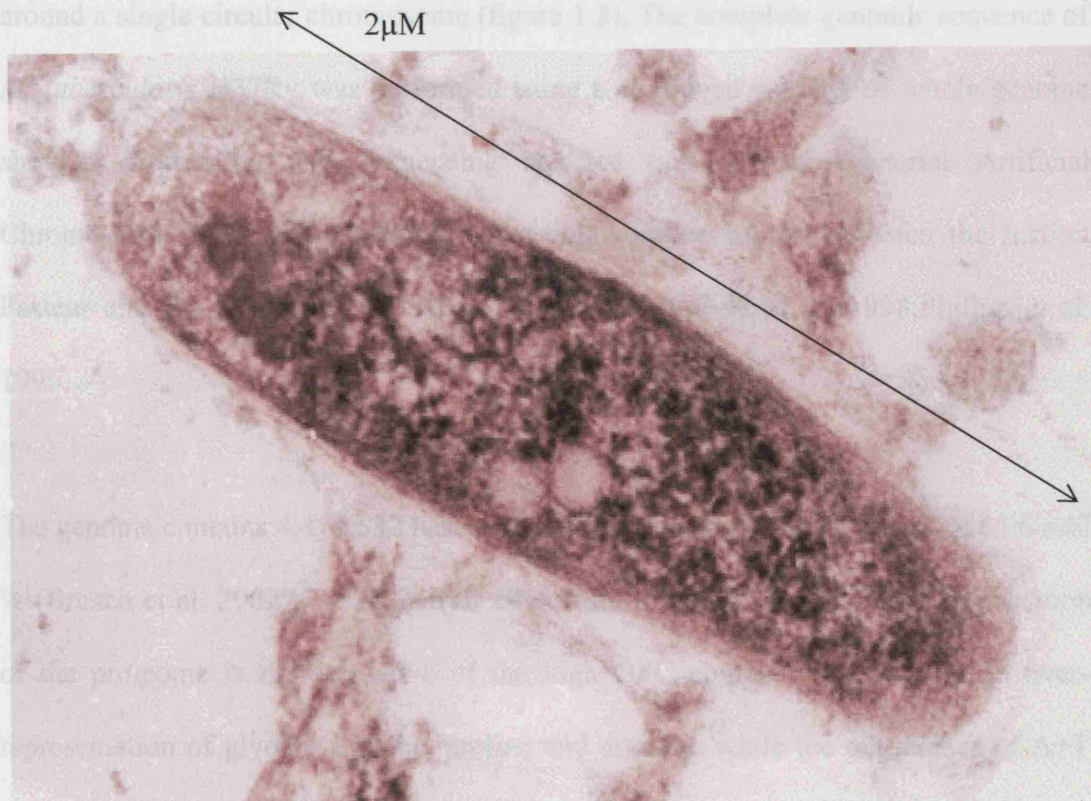
**Figure 1.1:** Phylogenetic tree of rapidly and slowly growing mycobacteria (Rastogi, Legrand, & Sola 2001).



### 1.3 Morphology

*M. tuberculosis* are straight or slightly curved non-motile, non-sporing, capsulate aerobic rods about 3 x 0.3µm in size (figure 1.2). They do not stain readily but when stained they resist decolourisation with tenacity (Gram positive) and are acid fast due to a lipid rich cell wall (Levy-Frebault & Portaels 1992). Mycobacteria are frequently stained with Ziehl-Neelsen (ZN) stain which is a mixture of the dye basic fuchsin, acid alcohol and methylene blue. They are resistant to digesting agents such as strong acids and bases but are sensitive to heat (pasteurisation) and UV light. They have a nutritional preference for lipids and so are frequently isolated on egg yolk medium such as Löwenstein-Jensen (LJ) medium which is often used in primary isolation of *M. tuberculosis* from pathological materials. The optimum growth temperature of tubercle bacilli is 35-37°C. They are slow-growing organisms and like other members in the *M. tuberculosis* complex divide every 18-24hr and take 2-3 weeks to form colonies on agar based media. Their characteristic slow growth is probably in part due to the hydrophobic nature of the cell surface, which renders the cells strongly impermeable to nutrients. They appear as off-white colonies, often with a dry crumb-like appearance and generally form tight compact colonies rather than spreading out over the surface of the agar. There is a difference in morphology between *M. tuberculosis* and *M. bovis* in that *M. bovis* have a more compact and raised appearance.

**Figure 1.2:** Thin section transmission electron micrograph of *M. tuberculosis* (Wadsworth Centre, NYS Dept. of Health). The length of the bacillus is indicated to show the scale.



## 1.4 The *M. tuberculosis* Genome

The *M. tuberculosis* genome has ~ 4000 open reading frames distributed fairly evenly around a single circular chromosome (figure 1.3). The complete genomic sequence of *M. tuberculosis* H37Rv was performed using a combined strategy of whole-genome shotgun sequencing and sequencing selected cosmid and Bacterial Artificial Chromosome (BAC) clones as part of a collaborative project between the Institut Pasteur and the Sanger Centre (Brosch et al. 1998; Cole et al. 1998; Philipp et al. 1996).

The genome contains 4,411,532 base pairs with a high mean G+C content of 65.6 mol % (Brosch et al. 2002; Cole & Barrell 1998; Cole 1999). The amino acid composition of the proteome is representative of the high G+C content composed of an over-representation of glycine, alanine, proline and arginine while the occurrence of A+T rich codons like lysine and asparagine are scarce. Approximately 52% of the genes have precise or putative functions assigned while the remaining 48% listed as conserved hypotheticals or unknown (Camus et al. 2002). Fifty genes encode stable RNA species and 3924 genes encoding proteins were identified. A significant portion of genes encode lipid metabolism (225 genes) (Cole 1999). Insertion sequence (IS) elements of which there are 56 copies make up 3.4% of the genome. These IS elements are composed of the IS3, IS5, IS21, IS30, IS110, IS256, ISL3 and IS1535 families. The most abundant IS element is IS6110, a member of the IS3 family. Restriction fragment length polymorphism (RFLP) typing of IS6110 has been the most widely applied molecular typing method for *M. tuberculosis* (Hermans et al.



1990;McAdam et al. 1990;Thierry et al. 1990), although new strategies are now being adopted (Kanduma, McHugh, & Gillespie 2003).

It is generally perceived that the *M. tuberculosis* genome is remarkably stable because of the rare occurrence of single nucleotide polymorphisms in coding regions (Musser, Amin, & Ramaswamy 2000;Sreevatsan et al. 1997a). Insertion and deletion events are regarded to provide the major source of genetic variation in *M. tuberculosis*. However single nucleotide changes may appear more polymorphic than originally thought as there appears to be substantial heterogeneity among *M. tuberculosis* clinical isolates. Kato-Maeda et al compared genomes within the species *M. tuberculosis* using high density arrays to detect deletions in strains. They found an average of 2.9 deletions per strain in 15 out of 16 *M. tuberculosis* clinical isolates (Kato-Maeda et al. 2001). However this heterogeneity in clinical isolates is less than is observed for other bacterial pathogens. The genome is dispersed with two protein families of simple sequence repeats referred to as MPTR (major polymorphic tandem repeat) and PGRS (polymorphic G+C rich sequence) which makes up the 3' end of protein families coding PE (proline-glutamate) and PPE (proline-proline glutamate) genes (Poulet & Cole 1995a;Poulet & Cole 1995b). PE and PPE proteins are two large exceptional glycine-rich protein families made up of 167 genes. Although asparagine is rare in the *M. tuberculosis* genome the PPE protein is unusual in that it contains large amounts of this amino acid which was shown to be the preferred nitrogen source for the mycobacteria (Grosset 1993).

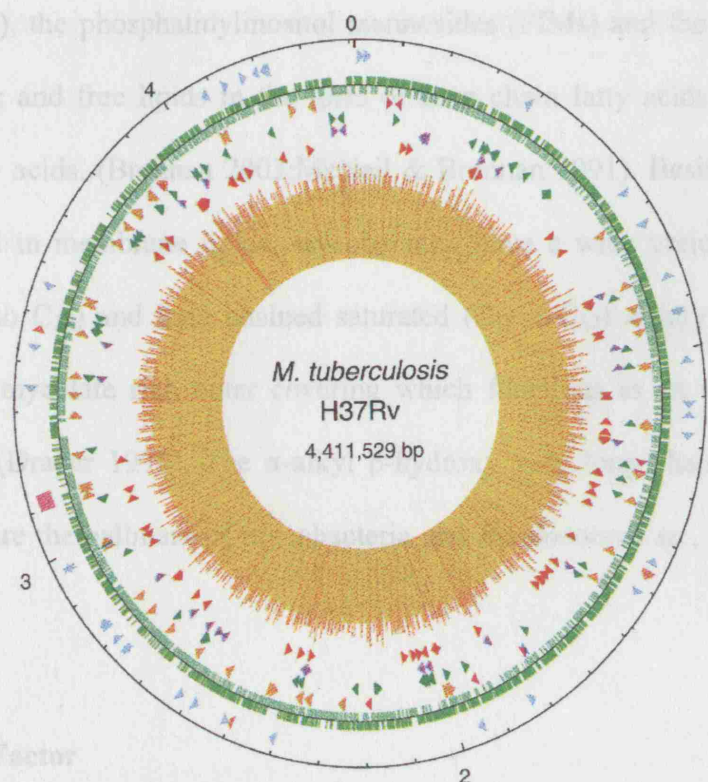
The completion of the *M. leprae* genome (3.27 Mb) shows that it is a severely contracted form when compared to the *M. tuberculosis* genome (4.4 Mb) (Cole &

Barrell 1998). *M. leprae* is a related obligate intracellular pathogen which has undergone extensive diversification (Philipp et al. 1996). Genes for anabolic functions are heavily conserved in this genome with the elimination of as many as 2600 genes (Cole et al. 2001; Eiglmeier et al. 2001), whereas *M. tuberculosis* employs catabolic pathways which is unusual for an intracellular pathogen to have retained such functions as the metabolites produced are scavenged by the host. This would suggest that *M. tuberculosis* has only recently emerged as a human pathogen as it had sufficient time to adapt to a new host which is evident by the retention of certain biosynthetic pathways.

The genome of *M. smegmatis* mc2 has been completed which is a mutant strain which is invaluable for analysis of mycobacterial gene replication, expression and function. The *M. fortuitum* genome is not being sequenced currently.

**Figure 1.3: The genome of *M. tuberculosis* H37Rv (Cole et al 1998)**

0 in the outer circle represents the origin of replication. The first ring from the exterior denotes the positions of tRNA genes in blue, other stable RNA genes in pink. The direct repeat region is represented as a pink cube. The second ring inwards depicts the coding sequence; the clockwise strand in dark green and anticlockwise strand in light green. The third ring shows insertion sequences in orange, 13E12 REP family in dark pink and prophage. The fourth, fifth and sixth rings show the positions of the PPE family members in green, the PE family members (excluding PGRS) in purple, and the PGRS sequences in dark red. The G C content is represented in the centre with a histogram (<65% G C in yellow and >65% G C in red).



## 1.5 Mycobacterial Cell Wall

### 1.5.1 Cell Wall Composition

Mycobacteria have a unique cell wall which is waxy, hydrophobic and has a high lipid content constituting 60% dry weight of the wall. It is composed of an upper and lower segment. The lower segment beyond the membrane is the cell wall core composed of a thin, inner layer of peptidoglycan in covalent attachment to arabinogalactan which is attached to the mycolic acids, collectively termed the mycolyl arabinogalactan-peptidoglycan (mAGP) (figure 1.4). The upper segment is composed of proteins and cell wall lipids which include; lipoarabinomannan (LAM), lipomannan (LM), the phosphatidylinositol mannosides (PIMs) and the phthiocerol-containing lipids; and free lipids in the form of long chain fatty acids interspersed with shorter fatty acids. (Brennan 2003; McNeil & Brennan 1991). Besides the usual fatty acids found in membrane lipids, mycobacteria have a wide variety of mono-unsaturated (up to C<sub>26</sub>) and long chained saturated (C<sub>18</sub> – C<sub>32</sub>) *n*-fatty acids. They produce a thick mycolate rich outer covering which functions as an exceptionally efficient barrier (Draper 1998). The  $\alpha$ -alkyl  $\beta$ -hydroxy very long chain fatty acids (mycolic acids) are the hallmark of mycobacteria and *Rhodococcus sp.*, among other bacterial species.

### 1.5.2 The Cord Factor

The growth pattern of *M. tuberculosis* in serpentine cords has led to the elucidation of cord factors, a surface mycolic acid-containing glycolipid, trehalose-6,6'-dimycolate.

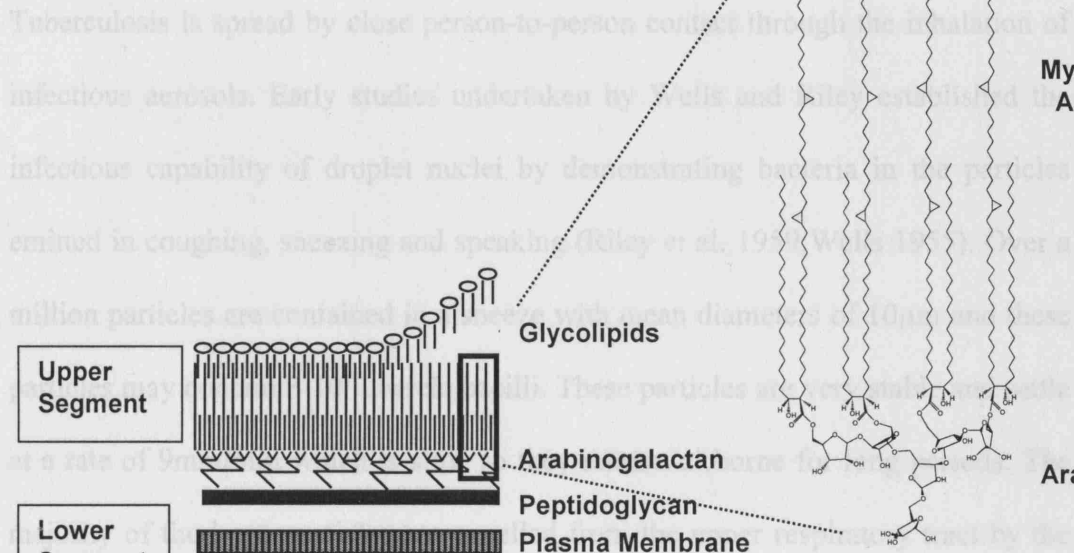
Middlebrook observed this growth pattern only in virulent strains of *M tuberculosis in vitro* (Middlebrook, Dubos, & Pierce 1947). Goren and Brennan studied cord factor and discovered various biological activities including the link between cord factor and NADase activity leading to lower NAD in tissues (Goren & Brennan 1980). Other activities include its anti-tumour properties, granulomagenic activity and immunogenic activities such as macrophage release of chemotactic factors (Matsunaga et al. 1990).

### **1.5.3 Characteristics of the Mycobacterial Cell Wall**

The cell wall is responsible for many of the characteristic properties of the mycobacteria including resistance to common antibacterial antibiotics, antigenicity and clumping (the cord factor). Disruption of the cell wall results in the solubilization of the free lipids, proteins while the mAGP complex remains as an insoluble residue. Isoniazid, a fundamental anti-tubercular agent, targets mycolic acid biosynthesis which can lead to this disruption (Dessen et al. 1995; Wheeler & Anderson 1996).

**Figure 1.4:** Cell wall of *M. tuberculosis*

## 1.6.1 Spread of Disease



While only 6% produces tubercles on reaching the alveoli (Smith & Moss 1994), *M. tuberculosis* has a very low infectious dose, estimated to be in the 10-100's of organism rather than the 1000's. It is said that a single person with active TB can infect many other people, but approximately 1 in 10 progress to develop active disease which explains its relative rarity (Vynnycky & Fine 2000). This risk of developing clinical disease is highest immediately after infection with an incidence rate of 4-5% in the first year, and then this decreases exponentially in subsequent years where the incidence for year 1-5 is 5-10% (Kearson & Boulton 1994). The progression time to develop primary disease is relatively slow, about 3-4 years (Sutherland 1968). The 10% rule is a good rough guide as to the likelihood of developing disease. However it does not take into account the nutritional status and/or living conditions and access to sufficient health care which can affect the

## 1.6 Epidemiology

### 1.6.1 Spread of Disease

Tuberculosis is spread by close person-to-person contact through the inhalation of infectious aerosols. Early studies undertaken by Wells and Riley established the infectious capability of droplet nuclei by demonstrating bacteria in the particles emitted in coughing, sneezing and speaking (Riley et al. 1959; Wells 1955). Over a million particles are contained in a sneeze with mean diameters of 10 $\mu$ m and these particles may contain 3-10 tubercle bacilli. These particles are very stable and settle at a rate of 9mm/min which is slow so they remain airborne for long periods. The majority of the large particles are expelled from the upper respiratory tract by the cilia while only 6% produce tubercles on reaching the alveoli (Smith & Moss 1994). *M. tuberculosis* has a very low infectious dose, estimated to be in the 10-100's of organism rather than the 1000's. It is said that a single person with active TB can infect many other people, but approximately 1 in 10 progress to develop active disease which explains its relative rarity (Vynnycky & Fine 2000). This risk of developing clinical disease is highest immediately after infection with an incidence rate of 1-5% in the first year, and then this decreases exponentially in subsequent years where the incidence for year 1-5 is 5-10% (Enarson & Rouillon 1994). The progression time to develop primary disease is relatively slow, about 3-4 years (Sutherland 1968). The 10% rule is a good rough guide as to the likelihood of developing disease. However it does not take into account the nutritional status and/or living conditions and access to sufficient health care which can affect the

progression of TB (Bloom et al. 1994). The life time risk of developing latent tuberculosis after infection is 90% (Hopewell 1994). Infection from the ingestion of tubercle bacillus as a result of eating contaminated food or using contaminated eating utensils rarely occurs as large numbers of bacteria must be ingested but they are very sensitive to gastric acid. It is estimated to be 10,000-fold less effective than the inhalation of aerosolized bacilli (Gaudier & Gernez-Rieux 1962).

Disease can develop as a consequence of inhalation of the bacilli directly from an individual with active pulmonary tuberculosis (progression of primary infection), exogenous reinfection (acquiring a new infection from another infectious individual) or endogenous reactivation of pre-existing 'dormant' infection (latent bacilli).

### **1.6.2 Global Burden**

It is estimated that, worldwide, 1/3 of the human population are infected with the *M. tuberculosis* bacillus (WHO 1999). Almost 2 million people died of tuberculosis in 2000 (WHO 2002b) with an estimated 8-9 million new cases, with case notification rates of less than 50%; 3-4 million cases were sputum-smear positive, the most infectious form of the disease (Corbett et al. 2003). The total number of cases of tuberculosis is rising slowly in the world at 1.7% a year but at varying levels across the globe (Dye 2003). If this trend continues, then by 2010 there will be 9-10 million new cases. The increase is due to inadequate case detection and treatment.



Eighty percent of cases reside in 22 high burden countries in developing areas of the world especially in South-East Asia, the western Pacific and Africa (Kurth & Haas 2002). Due to the HIV/AIDS epidemic, an increase of 10% of cases a year is observed in high burden countries especially in sub-Saharan Africa which has the highest incidence rate (290 per 100,000 population in 2000) but Asian countries with dense populations have the largest number of cases (3 million cases in the South East region): India, Bangladesh, Indonesia and Pakistan. Half the new cases in 2000 were in the top 5 countries all in Asia (Corbett et al. 2003). There has been a decrease in the rates of tuberculosis in western and central Europe, North and South America and the Middle East in the last 50 years and it has remained low in the past decade (Nguyen, Gilbert, & Marks 2004).

The resurgence of tuberculosis has been reported throughout Eastern Europe but especially in Russia where the increase has been the steepest (WHO 2000). In 1999, approximately 120,000 new cases (85 per 100,000 persons) were reported in the former Soviet Union with 30,000 deaths (20 per 100,000 persons) (Shilova & Dye 2001). This was 13,000 more cases than in 1998. The reasons for this sharp increase may be due to economic decline and the general failure and deterioration of tuberculosis control services (Shilova & Dye 2001). The tuberculosis death rate increased sharply in August 1998 due to temporary drug shortages because of the occurrence of a financial crisis. However, these drug shortages are experienced constantly due to inadequate funds for tuberculosis control. Risk factors exist in abundance in crowded prisons as well as in civil society. In 1999, the incidence of

tuberculosis in prisoners was reported as being 40 times higher than in the general population (3447 per 100,000 prisoners) with mortality being 100 times higher (238 per 100,000 prisoners) (Shilova & Dye 2001). Economic decline resulting from high labour turnover, unemployment, stress, war and declining social structure and equality is likely to have contributed to the increase of the tuberculosis incidence rates in Russia (Fedorov 1999; Stone 2000; Walberg et al. 1998).

### **1.6.3 A Tuberculosis Epidemic**

Various mathematical models have been proposed to estimate the doubling time of an epidemic of tuberculosis (Blower et al. 1995). The number of cases that arise from a primary case is low, an estimate of 2 cases in an untreated population (Dye & Espinal 2001). The estimated doubling time is 4-5 years for an epidemic in its early stages which is slower than most infectious diseases such as malaria or HIV.

### **1.6.4 Case Detection**

Case detection of tuberculosis is poor with the World Health Organisation (WHO) being notified of 40-45% of the estimated number of cases (Dawson 2001; Dye et al. 1999; Riley et al. 1959; WHO 2000; WHO 2002a). In Cuba the incidence rates of tuberculosis increased three fold from 1991 to 1994 due to economic difficulties and poor case finding. This increase was reversed in 1995 by improved case finding and the administration of effective chemoprophylaxis (Marrero et al. 2000). More than 98% of all deaths occur in developing countries where more than 95% of all

cases occur (Kurth & Haas 2002). The relative high case fatality of tuberculosis is due to untreated or improperly treated cases.

### **1.6.5 Risk Population**

Tuberculosis continues to kill more youth and adults than any other infectious disease. Globally, most cases (5-6 million) are in people aged 15-49 years. Tuberculosis is primarily a disease of adults and in particular males (Dye 2003). Ten percent of cases occur in children. In countries where transmission rates are high, for example in Peru, Haiti and Bolivia, the incidence of cases peaks in young adults. As transmission rates decline, the average age of disease increases; so in some countries with low transmission rates the elderly population have the highest occurrence of indigenous tuberculosis cases (Lillebaek et al. 2001; Llewelyn et al. 2000). In a country with a steady decrease in the incidence of tuberculosis (about 9 cases per 100,000 population), it is evident that tuberculosis is a disease of the elderly with an incidence of 4-8 times higher for people over 68 years when compared to below 30 years (Deutsches Zentralkomitee zur Bekämpfung der Tuberkulose 2002).

Generally tuberculosis is a disease of the poor, less developed countries suffering from over-crowded living conditions and inefficient healthcare systems but the situation can be exacerbated by national disasters, conflict and political instability (Roche et al. 2001). Tuberculosis is one of the main causes of mortality and morbidity among refugees and asylum seekers in camps (Houston 1998). In

Somalia, a study found that 26% of adult deaths among refugee populations was due to TB (Toole & Waldman 1988). WHO estimates that the occurrence of the disease among refugee camps to be about 4 times greater than in the local host population (WHO & United Nations High Commission for Refugees 1997).

#### **1.6.6 The Impact of Immigration**

In 2003, there were 14,874 cases of TB in the United States (CDC 2004). The number of TB cases in immigrants to the USA has not decreased for many years (Bloom 2002). As a result, immigrants infected with TB in their countries of origin are responsible in part for the further transmission and outbreaks of disease in the countries they immigrate to (Borgdorff et al. 1998; Lillebaek et al. 2002; Murray 2002).

Camie et al reported an increase in tuberculosis cases in the foreign born persons in Australia, even though the national incidence of tuberculosis has remained constant since 1986 (5-6 cases per 100,000 population) (Camie et al. 2001; Gilroy, Oliver, & Harvey 1998). Even though the impact of immigration must be taken into consideration, the transmission of TB between and within different ethnic groups is unknown. In Netherlands, Wolleswinkel-van et al designed a model based system to estimate the impact of immigration on transmission rates of tuberculosis within the country (Wolleswinkel et al. 2002). They estimated that by the year 2030, smear positive cases would not be eliminated and  $\geq 60\%$  of cases in the indigenous population would be as a result of transmission from foreign born cases.

In Western Europe, multiple molecular studies into the epidemiology of these countries have shown that there is little evidence that tuberculosis has spread from foreign born people to the indigenous population and the isolates among the non-indigenous people are more likely to be unique than clustered strains (Alland et al. 1994; Borgdorff et al. 2000; Hopewell 1994). A study into transmission rates in London between 1995 and 1997 have shown rates to be 14.4% with cases of tuberculosis largely caused by infected immigrants, reactivation of disease or newly acquired infection in the population with recognised risk factors (Maguire et al. 2002). In Germany about 30% of all new cases occur in the foreign-born population (Deutsches Zentralkomitee zur Bekämpfung der Tuberkulose 2002).

#### **1.6.7 Risk Factors Leading to Disease**

The most important risk factor increasing the likelihood of *M. tuberculosis* infection leading to disease is HIV infection. This factor accounts for the much of the recent increase in the global tuberculosis burden where 11% (612,000 out of 8.3 million cases) of all new tuberculosis cases in adults (15-49 years) in 2000 occurred along with HIV infection, of which 9% can be directly attributable to HIV (Corbett et al. 2003). However this varies substantially by region where in Africa (mainly sub-Saharan) 31% of new cases are attributable to HIV infection and in the United States 26% are (Corbett et al. 2003). HIV not only increases the risk of reactivating latent *M. tuberculosis* infection, it also increases the risk of developing rapid progressive disease upon primary infection. The increase in the risk of developing

rapid disease becomes more likely as there is an increase in the pool of people with latent infection. The risk becomes greater than 10% in the immunocompetent (Bucher et al. 1999; Girardi et al. 2000). The prevalence of *M. tuberculosis*-HIV coinfection in adults was 0.36% (11 million people) in 2000 and the higher the prevalence of HIV infection among smear positive TB cases the greater the contribution of HIV to tuberculosis transmission (Corbett et al. 2003). In South Africa alone there were 2 million co-infected adults (Corbett et al. 2003).

Other risk factors associated with the increase in the progression from infection with tubercle bacilli to development of tuberculosis are alcoholism (Rieder 1999), stress (Comstock 2000) and malnutrition (Palmer, Jablon, & Edwards 1957). It is commonly associated with the homeless, the elderly, the drug abusers and the health care workers who come in close contact with diseased patients.

In the United States, 16,377 cases (5.8 cases per 100,000 population) were reported to the CDC in 2000. This was a 45% decrease after a peak in the case rate in 1992, due to the epidemic of HIV infection, immigration, a deteriorating health care infrastructure and urban crowding (Division of the Tuberculosis Elimination 2001). The cost of the loss in control of the disease is estimated at costing New York City alone \$1 billion (Frieden et al. 1995).

### **1.6.8 Impact of Directly Observed Therapy – Short Course**

The World Health Organisation has promoted a treatment strategy known as DOTS – Directly Observed Therapy Short Course which is the cornerstone of tuberculosis control. The concept of supervised administration of medicines was first conceived by Wallace Fox (Fox 1958). The regimen can cure over 90% of new drug susceptible strains which can be a pre-requisite to increased case detection. The global target set by WHO for 2005 for case detection is 70% and treatment success is 85%. In 2002 the global smear positive detection rate was 37% which is only half way to the target (WHO 2004). By 2005, it is estimated that the detection level will be 50% but at present rates of DOTS expansion it will take until 2010 to reach the 70% target (Dye 2003). The WHO strategy was adopted by 75 countries by the end of 1995 but only 39 countries had implemented it nationwide (Raviglione et al. 1997). The total number of countries participating in the DOTS scheme increased to 180 out of 210 in 2003. It is estimated that 69% of cases reported reside in the countries covered by the DOTS program. In Peru, DOTS was launched in 1990, high rates of detection and cure have forced down the tuberculosis incidence by at least 6% per year (Suarez et al. 2001).

There are 5 essential elements (2 technical and 3 managerial) of the DOTS strategy. The technical elements are case detection by bacteriological examination in patients with symptoms self-reporting to health services followed by the administration of short-course chemotherapy by direct observation for at least the first 2 months of treatment. The managerial elements are securing a regular supply of essential

antitubercular agents, government commitment to mobilise resources for tuberculosis control and to allow assessment of treatment results, a standard reporting and recording system (WHO 1994). To increase case detection and cure rates, further interventions from governments must occur as the DOTS strategy alone is not enough. The total cost estimated for high burden countries (22 in total) rose from US\$834-884 million in 2002 to US\$1 billion in 2003 (WHO 2004). Impressively, between 1995 and 2002, a total of 13.3 million tuberculosis patients and 6.8 million smear positive cases were treated in DOTS programmes. The biggest challenge facing DOTS is the tuberculosis and HIV co-epidemic and the emergence of multi-drug resistant tuberculosis (MDR-TB). The most effective way to address the HIV-associated tuberculosis is by combining anti-retroviral therapy with DOTS rather than having one treatment in competition with the other.

#### **1.6.9 Epidemiological Molecular Tools**

Molecular tools to aid in the investigation of the epidemiology of tuberculosis have been developed to trace the origin of bacterial isolates from different patients. The *IS6110* element is the most widely used probe for RFLP typing which visualises restriction fragments that contain a repetitive sequence of DNA complementary to the probe (Thierry et al. 1990; van Embden et al. 1993). The identification of this insertion sequence element has been the standard genotyping technique for *M. tuberculosis* but there are several methods being adopted such as mycobacterial interspersed repetitive units (MIRU), polymorphic guanine cytosine-rich repetitive sequence (PGRS), MIRU-variable numbers of tandem repeat (VNTR) and



spoligotyping (Kanduma, McHugh, & Gillespie 2003). Supply et al demonstrated 41 loci with variable numbers of tandem repeats and 12 of these loci have formed the basis of the PCR-based typing method (MIRU) which vary in tandem repeat numbers and, in most of them vary in the sequence between repeat units (Supply et al. 2000). This method is better for low *IS6110* copy number strains compared to RFLP. Spoligotyping (for spacer oligotyping) analyzes a single locus to characterize *M. tuberculosis* (Kamerbeek et al. 1997).

## 1.7 Pathogenicity

### 1.7.1. Development of Disease

The bacteria's waxy outer coat enables them to withstand drying and to survive in dust and in the air after they are coughed up by infected individuals. The bacilli are susceptible to ultraviolet light. Four stages have been identified to describe the chronological order of the development of the clinical disease of tuberculosis (Dannenberg & Rook 1994).

The first stage is the inhalation of the tubercle bacilli (figure 1.5). These aerosolized infectious particles travel to the primary site of infection which is usually the lung (Chaparas 1982). The particles must be of an appropriate size to be deposited in the terminal airspaces of the lung (1-5µm in diameter) (Ferguson & Schlesinger 2000) and the droplet nuclei contain one to three bacilli (Wells 1955). The bacilli are then ingested by the alveolar macrophages (Lurie 1964). The bacteria may be destroyed by the macrophages but this depends on the intrinsic microbicidal activity of the macrophage and the virulence of the bacteria. The majority of individuals, approximately 90%, infected with *M. tuberculosis* control their infection and do not develop active disease (Comstock 2000).

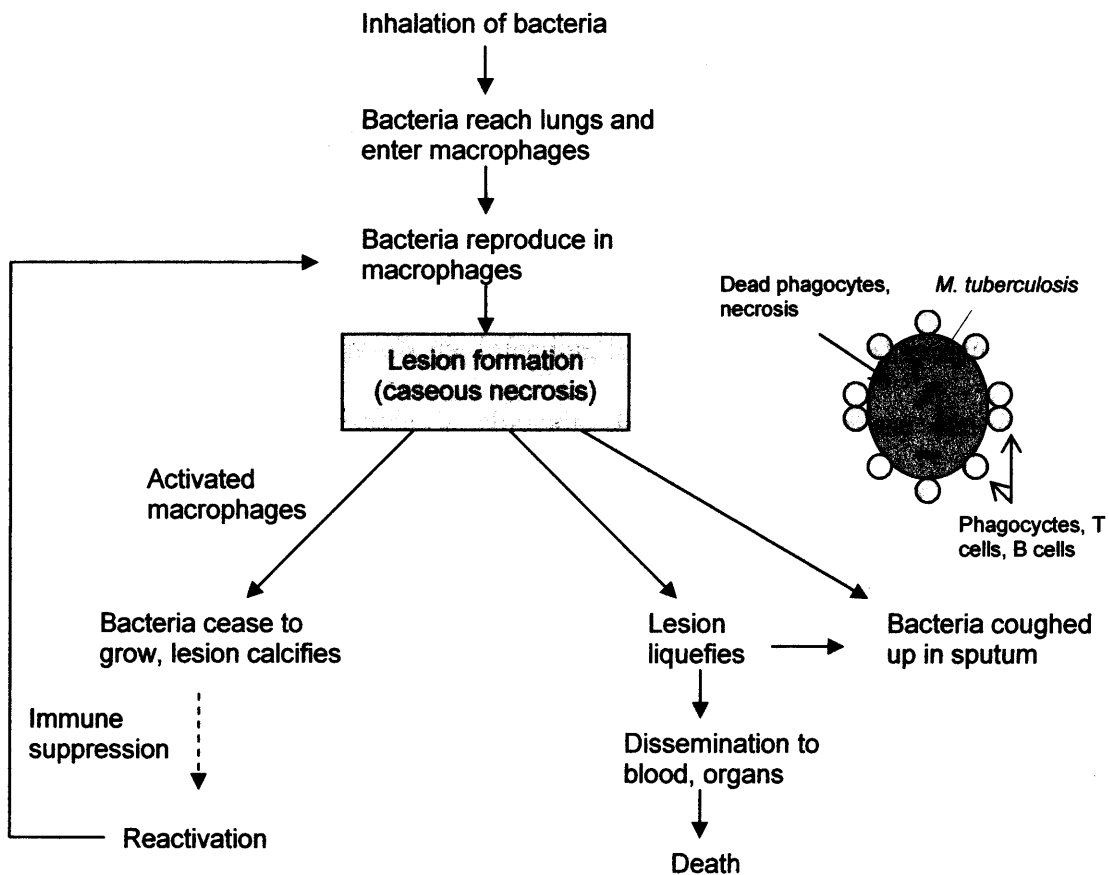
The second stage is initiated by the bacteria that escape the initial killing which then replicate within the macrophage, at the initial pulmonary site of infection (figure 1.5). The bacilli multiply until the macrophage bursts to release the progeny which are then ingested by other alveolar macrophages and also non-differentiated phagocytes such

as blood monocytes, among other inflammatory cells. This is the start of a symbiotic relationship between the tubercle bacilli and the new immature macrophages which have developed from the blood monocytes (Allison, Zappasodi, & Lurie 1962). It is within these blood-derived macrophages that the bacilli multiply logarithmically, for approximately 1-3 weeks after infection. Coinciding with this process T-cell immunity develops as antigen-specific T lymphocytes arrive to the site of multiplication. This population of T lymphocytes proliferates within the early lesions or tubercles, although relatively little tissue damage has occurred so far. It is this population of immune cells that activate the macrophages to kill the intracellular mycobacteria, thereby stopping the early log phase of growth. This is known as the third stage (initial caseous necrosis). The bacteria at this stage are within solid caseous material in the primary lesions where they are unable to multiply (Hemsworth & Kochan 1978). As a result infection may become stationary. Straight after primary infection, disease can progress by hematogenous dissemination. However this progression can take months to years to occur (postprimary tuberculosis) and is more likely during a period of poor immune surveillance by the host. Infection can spread to local lymph nodes within the lung and the progression of disease leads to cavity formation and liquefaction of the caseous lesions. The liquefied material within the caseous foci provides an excellent environment for the extracellular growth of *M. tuberculosis*. Cavity formation can lead to the rupture of nearby bronchi allowing eventual dissemination to other sites of the lung and remote sites of the body. Sometimes this can lead to acute, fatal disease in the form of tuberculous meningitis or miliary tuberculosis. Granulomas can form in any tissue which is infected with *M. tuberculosis* and are the characteristic histopathologic lesions of tuberculosis. They are composed of a compact cluster of T cells, macrophages and multinucleated

Langhans giant cells and contained within the centre is a mixture of necrotic tissue and dead macrophages which is referred to as a caseation, because of its cheese-like appearance and consistency (Saunders & Cooper 2000). The function of the granuloma is to segregate the infection to prevent spread to the remainder of the lung and to other organs and to enhance the immune response to concentrate at the site of infection. Replication of the bacillus is inhibited by the activated macrophage and subsequent killing is induced from the anoxic and acidic environment within the granuloma. This response is usually enough to prevent the development of primary disease. However, not all bacilli are destroyed and they persist in the body until possible reactivation. This reactivation results in postprimary tuberculosis and can occur when the patients immunological response wanes as a result of old age or therapy or development of an immuno-suppressive infection e.g. HIV.

Although the immune system is highly effective in targeting *M. tuberculosis* the organism is almost never eradicated, due to its capacity to establish and maintain a persistent but non-replicating state (a possible latent phase) (Glickman & Jacobs 2001). The balance between replication and killing of *M. tuberculosis* and the extent of necrosis, fibrosis and regeneration determines the final outcome of disease.

**Figure 1.5:** Pathogenesis of disease by *M. tuberculosis*



## 1.7.2 Latency and Reactivation

### 1.7.2.1 Development of Latent and Reactivation Tuberculosis

Development of disease is more commonly caused by the reactivation of a dormant *M. tuberculosis* infection which patients have carried for years rather than as a result of primary infection (Saunders & Cooper 2000). Approximately one third of the world's population (2 billion people) harbour quiescent *M. tuberculosis*. In 90% of the cases the immune system controls the infection, leaving the individual symptom-free and non-infectious. The disease is clinically latent and this phase can persist throughout the individual's lifetime. However there is a lifetime risk of developing reactivation disease of between 2% and 23% (Gedde-Dahl 1952). Several factors can influence the development of active disease such as HIV infection, malnutrition, cancer, diabetes, immunosuppressive drug therapy, drug use and chronic renal insufficiency (Parrish, Dick, & Bishai 1998). The risk for reactivation of the disease among HIV infected individuals is as high as 10% per year (Selwyn et al. 1989). The clinical diagnosis of latent tuberculosis is of a patient who displays the presence of a T lymphocyte-mediated immune reaction against a dermal injection of mycobacterial antigens, PPD or a chest radiograph that demonstrates scars indicative of old tuberculosis (Gedde-Dahl 1952).

'Latent tuberculosis' has been the subject of several reviews but it must be noted that is the disease that is latent rather than the bacilli themselves. Wayne and Sohaskey refer to this physiological state of the bacilli as a state of non-replicating persistence (NRP) (Wayne & Sohaskey 2001). Treatment of individuals harbouring latent

infection with isoniazid has shown a reduction in the risk of developing reactivation disease (Comstock, Baum, & Snider, Jr. 1979; Comstock & Woolpert 1972). Therefore the bacteria retain some replicative capacity during latency as susceptibility to antibiotics requires some level of metabolism and cell turnover. High dose rifampicin kills most non-actively replicating bacteria. A small proportion of the bacilli persist in lesions and this sentinel population of bacteria can tolerate the bactericidal effects of all the anti-tubercular agents and can escape the host's immune response (Hu & Coates 2003).

The presence of non acid fast forms of bacilli support the view that the persistent bacilli are in a spore like state, metabolically inactive. There are homologues of genes involved in the regulation of sporulation in *M. tuberculosis* such as *sigF* with similarity to SigF sporulation sigma factor in *Bacillus subtilis* (DeMaio et al. 1996). Also the fact that the bacteria may exist in a metabolic inactive state may mean that these organisms remain essentially invisible to the host immune system for such long periods of time. The issue of latency and drug resistance is a very important one as patients latently infected harbour protected populations within tubercle lesions and granulomas. It is these persistent populations which are protected against the bactericidal effects of anti-tubercular agents. Therefore the treatment of latent tuberculosis is crucial to prevent the emergence of drug resistance.

#### **1.7.2.2 Location of Persistent Bacilli**

Contained within the caseous foci, tubercle bacilli can persist in a dormant and non-metabolising state and are resistant to most other sterilizing anti-tubercular agents.

Little is known about this apparently latent or dormant phase of growth, including how the bacteria persist within the macrophages for decades asymptotically. Live bacilli have been isolated from the granulomas or tubercles in the lungs of persons with an absence of disease symptoms (inactive tuberculosis) which indicates that the bacteria are capable of persisting within granulomatous lesions for long periods of time (Opie & Aronson 1927;Robertson 1933). However microscopic examinations of tissues harbouring latent tuberculosis infection sometimes fail to identify acid-fast bacilli which has led to the hypothesis that latent bacilli are no longer acid fast due to an altered developmental state (Khomenko 1987;Stanford 1987). Perhaps the latent bacilli remain acid fast but are below microscopic detection levels. It is assumed that the latent bacilli are contained within the tuberculous lesions. Live bacilli have been reported in fewer than 10% of old lesions, while organisms have been reported in almost 50% of normal lung tissue from patients who had died from other causes than tuberculosis (Opie & Aronson 1927). But in patients that lack lung disease, reactivation must have originated from an extra-pulmonary site, so bacilli must have been spread to these locations during early exposure. Hernández-Pando et al showed the persistence of *M. tuberculosis* DNA in alveolar and interstitial macrophages which were the most common, but also in other non-professional phagocytic cells such as endothelial cells, fibroblasts and type II pneumocytes (Hernandez-Pando et al. 2000).



## 1.8 Clinical Presentation of Tuberculosis

Primary tuberculosis may be asymptomatic, presenting only on screening or on routine chest radiography. In 95% of infected immuno-competent patients, primary infection usually passes unnoticed and the primary complex resolves spontaneously (Grange 2003). Theoretically infection can occur at any site in the body; however the initial focus is primarily the lung which results in a dry, persistent cough. This is by far the most common symptom of bacteriological confirmed cases of tuberculosis. A study in Hong Kong showed that 81% of cases presented with a cough with only 15% having sputum production (Banner 1979). There are symptoms of fever, night sweats, dyspnoea (shortness of breath), chest pain and weight loss. Local complications of primary disease at the site of the primary lesion can include lobar collapse and hyperinflation, with pleural effusion also likely. Post primary disease occurring in individuals previously sensitised can lead to lung destruction with characteristic radiological features of soft fluffy shadows in the upper lobes with cavitation (Davies 2003).

Non-respiratory tuberculosis can manifest in the lymph node, bones and joints, gastrointestinal tract, genitourinary tract, bloodstream (miliary tuberculosis), the central nervous system and the pericardium (Ormerod 2003). Nodal enlargement is a symptom of lymph node tuberculosis. Early symptoms of CNS tuberculosis are non-specific but further stages including meningeal inflammation are accompanied with low grade fever, some neck stiffness, drowsiness or irritability. Miliary tuberculosis can be acute or cryptic, which is a form without typical x-ray shadowing which can be accompanied with weight loss, lethargy and intermittent fever (Proudfoot et al. 1969).

Between 6 and 8% of cases of extrapulmonary tuberculosis in England and Wales are due to miliary tuberculosis (Kumar et al. 1997), where 22% of cases of tuberculosis in white patients have a non-respiratory site. These were findings in a national survey of England and Wales in 1993. The proportion of tuberculosis at extrapulmonary sites has risen in the UK and other developed countries which may be due to immigration from developing high prevalence countries and HIV co-infection.

## 1.9 Diagnosis of Tuberculosis

### 1.9.1 Detection of *M. tuberculosis* in Clinical Isolates

The usual procedure for the identification of *M. tuberculosis* in clinical specimens is decontamination and digestion of the specimen, microscopic examination and isolation by culture of acid-fast bacilli and identification and drug susceptibility testing of the organisms recovered from culture (Soini & Musser 2001). Mycobacteria are cultured *in vitro* on egg based media such as LJ slopes as well as on solid Middlebrook 7H10 and 7H11 agar or in liquid 7H9 media. Due to the long incubation period required for growth of *M. tuberculosis*, clinical specimens may take 4-6 weeks to become positive. A staining method developed by Franz Ziehl and Fredrich Neelsen (ZN stain) takes advantage of the acid fast property of mycobacteria (Ziehl 1882). Organisms which are truly acid fast will retain the carbol-fuchsin in the face of decolourisation with acid-alcohol solution. This test does not differentiate between *M. tuberculosis* and non-tuberculosis mycobacteria. High performance liquid chromatography (HPLC) can be used to analyse mycolic acid content to identify mycobacterial species (Glickman et al. 1994) as can a range of molecular tools (Kim et al. 1999). When virulent *M. tuberculosis* is grown in liquid medium it sometimes forms characteristic serpentine cords which can be often used in its identification (McCarter, Ratkiewicz, & Robinson 1998). It has been indicated that only 50-80% of patients will have positive smears (DSCTAC 2000).

Culture is about 500 times more sensitive than microscopy and it generates a sample for further genotypic analysis (Huggett, McHugh, & Zumla 2003). Microscopy would

typically be able to detect acid fast bacilli in 60-70% of culture positive respiratory specimens with a minimum number of  $10^4$  bacilli per mL required for ZN staining (Garg et al. 2003), therefore making smears insensitive.

Some serological tests for tuberculosis include the use of enzyme linked immunosorbent assay (ELISA) which can detect antibodies to various purified or complex antigens of *M. tuberculosis*. These tests may not be able to distinguish between infection and disease and can lack sensitivity. Based on the same principles as ELISA, enzyme linked immunospot assay (ELISPOT) can be used to determine T cell reaction to a specific antigen with a very high sensitivity.

### **1.9.2 Liquid Culture Methods**

Culture methods may take 6-8 weeks to produce a result so liquid culture methods have been designed to increase the speed of diagnosis. The BACTEC 460 system is an automatic radiometric system using liquid cultures. It converts  $^{14}\text{C}$  labelled palmitic acid in the broth to radiolabelled  $^{14}\text{CO}_2$  which is then detected by the instrument (Watterson & Drobniewski 2000). This system has been superseded by non-radiometric systems such as MB/BacT (BioMerieux), BACTEC 9000 (Becton Dickinson) and the mycobacterial growth indicator tube (MGIT; Becton Dickinson). MB/BacT is adapted from the colorimetric system of detection of bacterial growth in blood cultures. MGIT can help in the early detection of mycobacterial growth and drug susceptibility testing using fluorochromes (Bemer et al. 2002; Tortoli et al. 1999).

### 1.9.3 Tuberculin Skin Test

The tuberculin skin test is a method for identifying *M. tuberculosis* in patients who have recently been in contact with an index case of pulmonary tuberculosis (Fitzgerald & Menzies 2003). The use of tuberculin in the skin test was first described in 1907 by von Pirquet after Robert Koch in 1890 developed an extract from heat killed *M. tuberculosis* to inject into guinea pigs which evoked a destructive inflammatory response at the site of injection (Koch 1890; von Pirquet 1907). Purified protein derivative (PPD) is now the accepted standard reagent. False positive results can occur due to prior sensitization with environmental mycobacteria or previous BCG vaccination. A negative tuberculin skin test in patients with tuberculous infection or disease can result from HIV infection, malnutrition or steroid therapy which all interfere with the Th1 delayed hypersensitivity response or active tuberculosis itself (Barnes 1994). Tuberculin conversion is a biological increase in tuberculin reactions as a result of a new mycobacterial infection and it can occur 3-7 weeks following new tuberculosis infection (Poulsen 1954; Youmans 1979).

### 1.9.4 Molecular Detection of *M. tuberculosis*

Molecular methods for the identification of *M. tuberculosis* can reduce diagnostic time from weeks to days. The majority of molecular tests are based on the polymerase chain reaction (PCR). The Food and Drug Administration (FDA) have approved two tests for the direct detection of *M. tuberculosis* from respiratory specimens: the AMPLICOR system targeting the 16S rRNA gene through conventional PCR from Roche (Roche Diagnostic Systems, Inc, Indianapolis, Ind.) and a system provided by

Gen-Probe (Gen-Probe, Inc, San Diego, California) which utilises transcription mediated amplification of the 16S rRNA (Woods 2001). PCR-based sequencing has become a universal method for the identification of mycobacterial species. The target used for identification can be insertion and repetitive elements, various protein encoding genes and of most use is the 16S rRNA gene. This gene is universal in bacterial and archeal species and is made up of conserved and non-conserved regions which make it an ideal target in differentiating between species. A commercially available PCR-based kit (LiPA, Mycobacteria; Innogenetics, Zwijndrecht, Berlin) can identify a range of species with specific probes. It targets the 16S-23S ribosomal spacer region and the probe-hybridised product bound to a nylon strip; the binding is identified colorimetrically. Strand displacement amplification (SDA Probetech, Becton Dickinson) is an *in vitro* nucleic acid amplification technique which utilises the restriction enzyme *HincII* to target DNA IS6110 using amplification primers and fluorescently labelled probes (Spargo et al. 1993). Direct amplification and sequencing of a range of loci associated with resistance to rifampicin, isoniazid, pyrazinamide, ethambutol and other antitubercular drugs can be a useful method (Musser 1995).

Nucleic acid amplification tests (NAAT) have aided the identification of drug resistant isolates of *M. tuberculosis*. A kit to identify mutations in the *rpoB* gene in *M. tuberculosis* to detect resistance to rifampicin is available commercially (INNO-LiPA Rif TB) which can identify 90-95% of resistant strains. The kit fails to identify mutations outside the rifampicin resistance determining region (RRDR) (Jenkins et al. 2005). A negative PCR result never eliminates possibility of tuberculosis and a positive result is not always confirmatory, other factors such as smear positivity and

risk factors are taken into consideration before a diagnosis is made. A good quality PCR should be highly sensitive; 90-100% on smear positive samples and 60-70% on smear negative, culture positive respiratory samples (Watterson & Drobniewski 2000). The main drawbacks with PCR are problems with cross-contamination and false positivity as a result of poor assay design (Gillespie & McHugh 1997).

## 1.10 Treatment

### 1.10.1 The Population Theory

It is believed that the most actively replicating bacteria are killed at the beginning of tuberculosis therapy and prolonged treatment is required to eliminate the persisting, nonreplicating organisms which have a reduced or altered susceptibility to the antituberculosis agents (Yamori et al. 1992). In figure 1.6 population 'a' represents the majority of bacilli which are in log phase, actively dividing in lesions of untreated patients and these cells are rapidly killed by isoniazid and to a lesser extent by rifampicin. A slower growing population (figure 1.6 population 'b') which may be in closed lesions where oxygen supply is limiting are killed effectively by rifampicin. Pyrazinamide kills a further slow growing population within the acidic environment of phagolysosomes within macrophages (population 'c'). It is estimated that this population located within solid caseous foci are low in numbers (Canetti 1955). Population 'd' are known as persisters as they are tolerant of anti-tubercular agents including rifampicin and are not killed by the immune system of the host (Hu & Coates 2003; Mitchison 1979; Mitchison 1997).

In another model system developed by Gillespie & McHugh, bacteria are considered which communicate within 3 compartments; bronchioles and alveoli, cavities or empyema, and a population only susceptible to pyrazinamide. It is estimated in this model that cure depends on the penetration of antibiotics into the bronchioles which they can penetrate readily (Personal communication Prof. S.H. Gillespie). There is no experimental evidence as to the penetration levels of antibiotics into the cavities but it

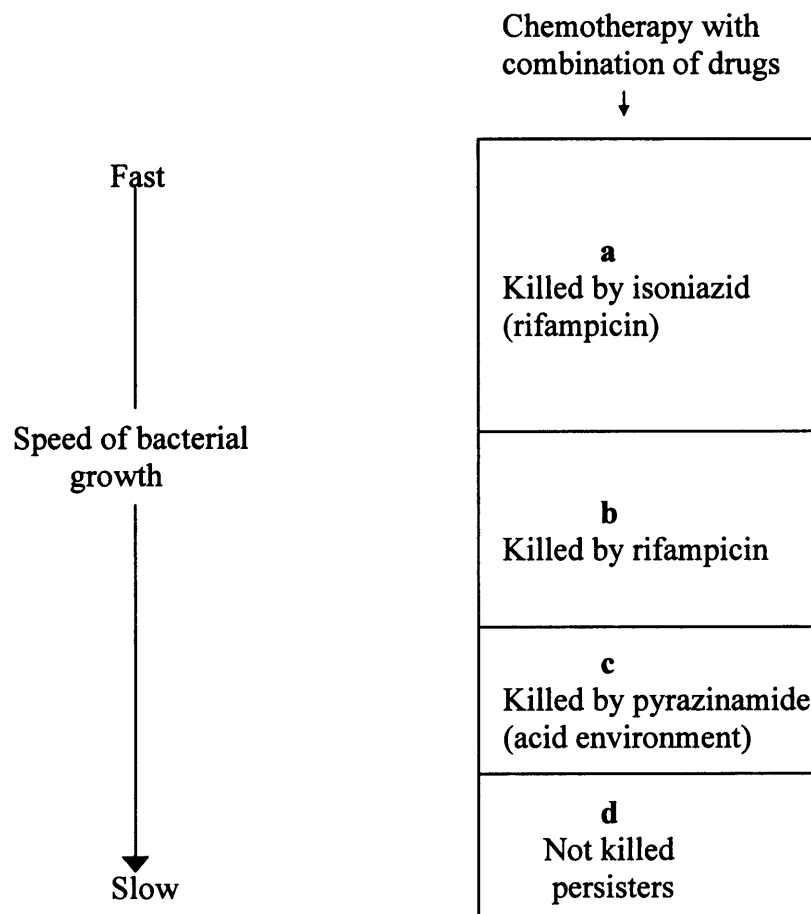


is thought to be reduced. Previous studies have related cavity size to patient outcome (East African/British Medical Research Council 1981).

**Figure 1.6:** Hypothetical picture of sub-populations of tubercle bacilli within lesions.

Adapted from Dormancy and Low Growth States in Microbial Disease Ed. A.R.M.

Coates. Chapter 7 (Hu & Coates 2003).



### 1.10.2 Treatment Regimens

Treatment using prolonged multiple drug regimens is required to prevent the development of drug resistance. WHO recommend treatment consisting of an initial intensive phase of 4 drugs for 2 months followed by 2 drugs for 4-6 months. Rifampicin and isoniazid are the two principal first line drugs used (Pio & Chaulet 1998) (table 1.1). Other drugs used are pyrazinamide and ethambutol with or without streptomycin for the initial phase of treatment (Chan & Iseman 2002). Streptomycin and ethambutol are weaker but nevertheless effective anti-microbials. The treatment regimen outlined by BTS guidelines for respiratory tuberculosis recommends a six month regimen comprising of rifampicin, isoniazid, pyrazinamide and ethambutol for the initial two months followed by rifampicin and isoniazid for a further four months irrespective of the bacteriological status of the sputum (Joint Tuberculosis Committee of the British Thoracic Society 1998).

Ethambutol can be omitted in patients with a low risk of primary resistance to isoniazid (Singapore Tuberculosis Service/British Medical Research Council 1985; Snider et al. 1984). These patients are previously untreated white, HIV-negative and who have no known contact of a patient with drug resistant organisms. The treatment for non-respiratory tuberculosis is the recommended 6 month regimen for respiratory tuberculosis. The DOTS strategy has been a very successful intervention in reducing the TB burden in some countries (Espinal et al. 2001). The impact it has had has caused a reduction in the number of tuberculosis cases in the 1990's in the United States, where there has been an increase of 70% in the patients receiving DOTS in 2000 from 4% (CDC 2000). In the UK, DOTS may be recommended for

individuals who are likely to not comply with treatment (homeless, alcoholic and drug abusers, vagrants, multi drug resistant patients, new immigrants and refugees). An intermittent regimen is more convenient for patients being treated under the DOTS regimen. This regimen to treat fully sensitive organisms includes isoniazid, rifampicin and pyrazinamide three times weekly for two months, followed by isoniazid and rifampicin three times weekly for a further four months.

Treatment failure rates are alarmingly high for pulmonary tuberculosis and have been reported as high as 67% (Walley et al. 2001). This is despite having effective agents in the treatment of tuberculosis. This is alarming as no new chemotherapeutic agents have improved treatment regimens in 30 years since the introduction of rifampicin and pyrazinamide (Combs, O'Brien, & Geiter 1990; Gillespie & Kennedy 1998; Ginsburg, Grosset, & Bishai 2003).

Chemoprophylaxis for tuberculosis infection is administered for asymptomatic individuals with a positive tuberculin skin test and normal chest radiographs. The treatment consists either of one drug for six months or two drugs for three months (Joint Tuberculosis Committee of the British Thoracic Society 1990).

**Table 1.1:** Recommended dosages of standard antituberculosis drugs. Adapted from: Chemotherapy and management of tuberculosis in the United Kingdom: recommendations 1998 (Joint Tuberculosis Committee of the British Thoracic Society 1998).

Drug	Weight of Adult	Daily Dosage for Adults
Isoniazid	-----	300mg
Rifampicin	<50kg	450mg
	≥50kg	600mg
Pyrazinamide	<50kg	1.5g
	≥50kg	2.0g
Ethambutol	15mg/kg	15mg/kg

### 1.11 Drug Resistance

*M. tuberculosis* has several strategies to protect itself against antimicrobial agents; as mentioned (section 1.5) the bacillus has a specialised hydrophobic cell wall which results in decreased permeability to many compounds making the bacteria resistant to therapeutic compounds such as penicillin and sulfa based compounds. Resistance in *M. tuberculosis* depends almost entirely on genetic alterations within the genome (Riska, Jacobs, Jr., & Alland 2000). It is thought that drug resistance arises due to dominant survival of random pre-existing mutations rather than drug-induced mutations. However it has been shown that treatment of *M. fortuitum*, used as a model system for *M. tuberculosis* with sub-MIC levels of quinolone can increase the mutation rate by up to 120 fold (Gillespie et al. 2005). MIC (minimum inhibitory concentration) is defined as the minimum concentration of an antibiotic in a given culture medium below which bacterial growth is not inhibited. Resistance develops as a consequence of spontaneous mutations in genes that encode the drug target or enzymes that are involved in activation of the drug. Intrinsic resistance can result from the activity of efflux pumps and the degrading or deactivation of enzymes. There are no plasmids present in *M. tuberculosis*. Although, IS elements are present, there is no evidence of these being involved in transmission of DNA and therefore no sudden development of multiple resistance in a strain takes place (Blanchard 1996). The only example of resistance developed through transposable elements in mycobacteria is sulphonamide resistance in *M. fortuitum* which occurs due to the transposition of a drug inactivation enzyme (Zhang & Young 1994).

*M. tuberculosis* resists killing when in stationary phase which allows it to persist (Wallis et al. 1999). Herbert et al demonstrated that the sterilising activity of isoniazid

is reduced nearly 1,000 fold in stationary phase cultures (Herbert et al. 1996). Even though spontaneous drug resistance occurs rarely in *M. tuberculosis*, there has been an increase in prevalence of drug resistant strains due to non-compliance with treatment and the growing number of immuno-compromised patients (March et al. 1997). Further understanding on a molecular basis of the mechanisms of drug resistance in tuberculosis will help in the development of improved screening and identification methods for determining whether strains are drug resistant.

Spontaneous chromosomal mutations (point mutations) arise at a low frequency among isolates of *M. tuberculosis*. For base substitutions, the rate for a specific base change in *E. coli* is in the order of  $10^{-10}$  -  $10^{-9}$  per base pair per cell generation (Fowler, Degnen, & Cox 1974; Mackay, Han, & Samson 1994). This is assuming that normal repair systems exist. However there are “hotspots” for spontaneous mutations where mutations occur at a higher frequency than would be predicted. In *M. tuberculosis* random mutations occur at a rate of  $10^{-6}$  per base pair per cell generation to isoniazid, 1 in  $10^6$  to streptomycin, 1 in  $10^6$  to ethambutol and 1 in  $10^8$  to rifampicin (Long 2000). Long estimated that a tuberculous cavity contains  $10^7$  –  $10^9$  bacilli, then about 10-1000 bacteria would be isoniazid resistant, 10-1000 streptomycin resistant, 10-1000 ethambutol resistant and  $\leq 10$  rifampicin resistant. On sampling this population, resistance would not be reported to all of the antituberculosis agents, as the resistance must be present in  $\geq 100$  bacilli to be detected and reported in the laboratory. Bacillary populations greater than  $10^7$  are common in lung cavities in infected patients. Thus in the absence of antimicrobial exposure, resistant organisms evolve but are diluted by the majority of drug susceptible mycobacteria. Upon exposure to antimicrobials, a selective pressure favouring a resistant cell occurs and

multiplies to become predominant. This situation is especially relevant in patients with extensive cavitation disease (i.e. have a large bacillary load) (WHO 2003).

Epidemiologically drug resistance can be described as being of 3 types: primary, acquired and initial (Long 2000). Primary drug resistance is when patients have been infected with drug resistant organisms from an outside source; acquired is when drug susceptible bacilli become resistant because of nonadherence to treatment protocols or inadequate, inappropriate or irregular treatment; initial applies to patients who are unsure of prior treatment history.

### **1.11.1 Rifampicin**

#### **1.11.1.1 Activity of Rifampicin**

Rifampicin is a semi-synthetic derivative of the natural product rifamycin and was introduced as an antitubercular drug in 1972 (Woodley et al. 1972). It is an extremely effective rapid bactericidal drug (Mitchison 1985). It has a broad antibacterial spectrum and a well defined target. Rifampicin has bactericidal activity against active and the near dormant bacilli that reside within macrophages as well as extracellular bacilli. It inhibits the initiation of transcription and elongation of the RNA (Gale et al. 1981; McClure & Cech 1978). However it was shown using purified RNA polymerase from *M. smegmatis* mc<sup>2</sup>155 that rifampicin specifically inhibited the elongation of full-length transcripts with practically no effect on transcription initiation (Levin & Hatfull 1993). RNA polymerase is an ideal chemotherapeutic target as it plays a

central role in genetic information transfer within bacteria and its inactivation will result in total inhibition of cellular functions.

#### 1.11.1.2 Rifampicin Resistance

Rifampicin resistance occurs through a mutation in the  $\beta$  subunit of RNA polymerase, encoded by *rpoB*, more specifically associated with mutations in the 81 base pair core region (Telenti et al. 1993) (figure 1.7). Within this 81bp region (from codons 507 through to 533), missense mutations, insertions and deletions are responsible for resistance in more than 95% of mutations in clinical isolates with the vast majority (93%) being single nucleotide changes. Other studies have revealed the mutations associated with resistance to rifampicin can occur outside the hotspot region in the amino-terminal region of *rpoB*, but these occur less frequently (Heep et al. 2001; Heep et al. 2000; Zhang & Telenti 2000). More than 35 mutations within this region have been reported (Musser 1995). More importantly, 43% of strains have a missense change at serine 531 (of which 42% were Ser531Leu) and 36% have a missense change at histidine 526 (of which 23% were His526Tyr) (Ramaswamy & Musser 1998). These mutations are absent in susceptible organisms. Morlock et al answered the question as to whether mutations within this hotspot region are a feature of clinical strains, when they reported the same phenomenon in spontaneous rifampicin resistant mutants of *M. tuberculosis* strain H37Rv (a laboratory strain) (Morlock, Plikaytis, & Crawford 2000). The degree of rifampicin resistance is dependent on the location of the mutation within the hotspot region with alterations at codons 526 or 531 resulting in high level resistance. Resistance to rifampicin occurs at a rate of  $10^{-8}$  per cell division (Werngren & Hoffner 2003), a rare event. More than 90% of rifampicin

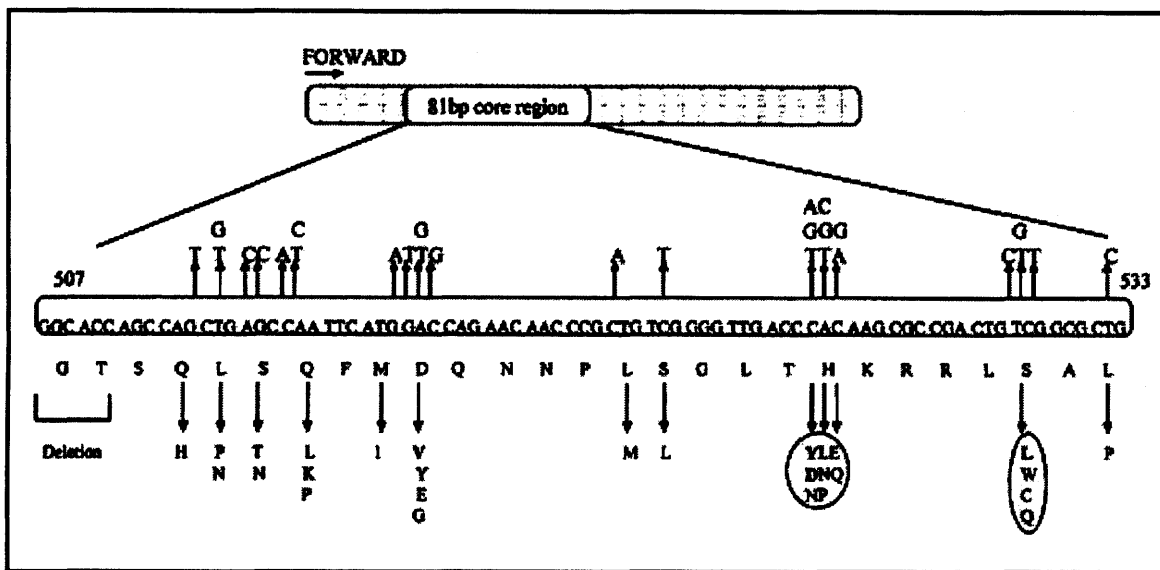


resistant isolates are also isoniazid resistant (Drobniewski & Wilson 1998). It is because of this that rifampicin resistance is an excellent marker for multidrug resistant TB and also because resistance is restricted to the rifampicin resistance determining region (RRDR).

#### 1.11.1.3 Rifampicin Derivatives

Newer synthetic derivatives of rifampicin such as rifabutin and KRM-1648 (benzoxazinorifamycin) are more active against *M. tuberculosis in vitro* and *in vivo* (Gangadharam et al. 1987; Ji et al. 1993). However there is evidence of cross resistance with rifampicin due to their similar mode of action (Yang et al. 1998). There is some evidence that rifabutin is active against rifampicin resistant tuberculosis and therefore useful as second line agent in therapy (Luna-Herrera, Reddy, & Gangadharam 1995). Alterations in codons 511, 516, 518 and 522 result in low-level resistance to rifampicin and rifapentin (rifamycin derivative), but remain susceptible to rifabutin and rifalazyn (another rifamycin derivative) (Moghazeh et al. 1996; Ohno et al. 1996).

**Figure 1.7:** Distribution of resistance mutations in *rpoB* of clinical isolates of *M. tuberculosis* occurring in codons 507 -533. The codon numbers are from the translated *E. coli rpoB* sequence and are not the actual positions in the *M. tuberculosis rpoB* (Rattan, Kalia, & Ahmad 1998).



### 1.11.2 Isoniazid

#### 1.11.2.1.a Activity of Isoniazid

Isoniazid was recognised in 1951 to be a powerful antitubercular agent (Fox 1951) which showed excellent activity against *M. tuberculosis*, *M. bovis*, *M. bovis* BCG and *M. africanum* with no activity against non-tuberculous mycobacteria (Wolinsky 1979). It has a high early bactericidal activity (EBA) which is the fall in the numbers of viable tubercle bacilli in sputum during the first few days of treatment (Gillespie, Gosling, & Charalambous 2002; Jindani et al. 1980). For this anti-tubercular agent the

pre-dominant action is in killing of actively growing *M. tuberculosis*. It has limited sterilising activity which is crucial in the killing of semi-dormant organisms which constitute the remaining bacilli (Mitchison 1993).

#### 1.11.2.1.b Catalase-peroxidase Activity

Isoniazid is a drug that requires activation by the bacterial enzyme catalase-peroxidase. KatG shows the typical structure of bacterial hydroperoxidase I catalase-peroxidases and is divided into two parts. The catalase activity in this enzyme converts  $2\text{H}_2\text{O}_2$  to  $2\text{H}_2\text{O}$  and  $\text{O}_2$  which plays an important role in the organism's capacity to deal with oxidative stress. The bi-functional nature of this enzyme means that its other activity in peroxidase mode accepts 2 electrons from organic donors to reduce  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$ . The peroxidase activity of this enzyme seems to be necessary for the activation of isoniazid to a toxic substance within the cell (Zhang & Young 1993). The activation of isoniazid is thought to result in the generation of highly reactive products, including electrophilic free radicals and peracid intermediates (Johnsson & Schultz 1994). Reactive oxygen intermediates, which are usually generated during oxidative respiration, are believed to damage various molecules in the cell; nucleic acids and unsaturated fatty acids are particularly vulnerable (Jacobson et al. 1989). Activated isoniazid inhibits mycolic acid synthesis, the main target (Takayama, Wang, & David 1972). This inhibition of mycolic acid synthesis is observed by a loss in acid fastness of the tubercle bacillus (Takayama, Wang, & David 1972).

### 1.11.2.2 Isoniazid Resistance

When treatment for tuberculosis fails in immuno-competent patients, resistance to isoniazid usually appears first (Elghoul, Joshi, & Rizghalla 1989). Resistance to isoniazid has been found to occur through mutations in the following genes: *katG*, *ahpC*, *kasA* and *inhA*, with mutations in *katG* and *inhA* associated with 70-80% of isoniazid resistant *M. tuberculosis* isolates (Rattan, Kalia, & Ahmad 1998). Resistance to isoniazid is a complex phenomenon and involves several mechanisms.

#### 1.11.2.2.a *katG*

Resistance to isoniazid occurs most frequently by mutation of *katG* resulting in diminished peroxidation of isoniazid to its active form (Heym et al. 1994; Musser et al. 1996; Pym, Saint-Joanis, & Cole 2002). Evidence shows that 40-50% of isoniazid resistant clinical isolates have missense mutations or deletions within *katG* (Zhang & Telenti 2000). Mutations in *katG* that alter or abolish the catalase-peroxidase activity of the bacteria can prevent the activation of the prodrug and lead to isoniazid resistance. Reduced uptake of isoniazid has been demonstrated in many isoniazid resistant, catalase negative *M. tuberculosis* strains (Youatt 1969), suggesting that KatG may facilitate the uptake of isoniazid into the bacterium. In organisms displaying isoniazid resistance, reduced catalase activity is observed (Zhang et al. 1992) and has also shown attenuated virulence in the guinea pig model (Middlebrook 1952).

Complete *katG* deletion has been shown in highly isoniazid resistant clinical isolates of *M. tuberculosis* (Zhang et al. 1992) but it rarely occurs (Altamirano et al.

1994;Cockerill, III et al. 1995;Pretorius et al. 1995;Stoeckle et al. 1993). This is not a consistent phenomenon as it does not explain resistant isolates with / without mutations in this gene and peroxidase activity in KatG is necessary to detoxify host antibacterial radicals (Dobner et al. 1997;Haas et al. 1997;Temesgen et al. 1997). Point mutations are more common than deletions of the entire gene (Pretorius et al. 1995;Rouse & Morris 1995). Deletions and insertions have been shown to be associated with isoniazid resistance (Altamirano et al. 1994). Isoniazid sensitive isolates have also been reported to contain mutations in their *katG* (Pretorius et al. 1995). The majority of mutations in *katG* are missense single nucleotide substitutions.

It has been shown that the most commonly occurring *katG* mutation is Ser315Thr (Pym, Saint-Joanis, & Cole 2002). Mutations at this position occurred in 49/85 (58%) isoniazid resistant strains (Musser et al. 1996). The Ser315Thr mutation at this amino acid retains approximately 50% of its catalase-peroxidase activity with the combination of decreased catalase activity but maintaining a certain level of peroxidase activity (Rouse et al. 1996) (Ramaswamy & Musser 1998). This provides a substantial advantage to the bacterium as it retains significant protection against host antibacterial radicals while providing high level resistance to isoniazid. The altered catalase-peroxidase activity confers high level resistance to isoniazid, while retaining the ability to detoxify host antibacterial radicals. Ser315Thr, Thr275Pro, Leu587Met, deletion of residues 120-123 and insertion of an isoleucine between residues 125 and 126 are all predicted to affect the functioning of catalase (Heym et al. 1995). All of these mutational events occur within the *N*-terminal domain of the enzyme which is believed to contain the active site residues, characteristic of peroxidase enzymes. Mutations within the *N*-terminal domain are associated with strongly reduced enzyme

activity and increased heat lability (Heym et al. 1995). The question of why only 10% of isoniazid resistant isolates lack catalase-peroxidase remains to be answered, despite the close relationship between production of the enzyme and susceptibility to isoniazid (Stoeckle et al. 1993). It is clear that a significant number of isoniazid resistant isolates retain reduced, but considerable, catalase-peroxidase activity. It is possible that retaining enzymatic activity confers a selective advantage for *M. tuberculosis* enabling it to withstand the environment within the macrophage or by allowing it to multiply more rapidly which has been observed in other mycobacteria devoid of the enzyme (Heym & Cole 1992). The *katG* mutations which reduce enzymatic activity appear to do so by two mechanisms: a reduction in KatG concentration and/or a direct effect on enzymatic function.

The point mutation at codon 463 of *katG* resulting in Arg → Leu variation was identified in 19 out of 43 (44%) *M. tuberculosis* strains (Cockerill, III et al. 1995; Musser 1995). This mutation represents a loss of a GGCC restriction site. It is situated outside the *N*-terminal domain at the end of the helix A in the *C*-terminal repeat and is thought to be outside the peroxidase binding site. Perhaps this location in the gene represents a substrate binding site to which isoniazid interacts with the enzyme (Heym et al. 1995). Mutations located in the *C*-terminal domain do not noticeably alter enzyme activity or heat stability. The importance of this mutation as a marker for isoniazid resistance has been confounded as it has also been identified in isoniazid sensitive strains.

#### 1.11.2.2.b *inhA*

It has been shown that mutations in *inhA* can confer resistance to both ethionamide and isoniazid and even been suggested that it is the primary target for coresistance to these agents (Banerjee et al. 1994). Ethionamide is a structural analog of isoniazid which is thought to prevent mycolic acid synthesis, in a similar manner to isoniazid (Winder, Collins, & Whelan 1971) and can be sometimes used as a second line antitubercular agent. Even though it is believed that isoniazid and ethionamide share the same target enzyme, cross resistance occurs in a minority of strains and *katG* plays no role in the case of ethionamide. The product of *inhA* is a protein involved in mycolic acid synthesis, more specifically is its involvement in an essential step in fatty acid elongation in the catalysis of the NADH specific reduction of 2-trans-enoyl acyl carrier proteins (ACP) (Dessen et al. 1995). Single nucleotide changes at position 94 in *inhA* resulting in a serine to alanine change confer resistance to isoniazid (Whitney & Wainberg 2002). Mutations in the adjacent codon 95 have been found in 7 out of 14 isoniazid resistant isolates from Nepal (Ristow et al. 1995). Kapur et al analysed 37 isoniazid resistant isolates and found no Ser94Ala substitutions (Kapur et al. 1995). In another study by Morris et al, no mutations were observed in *inhA* among 42 isoniazid resistant isolates (Morris et al. 1995) and in a subsequent study the same findings were observed in 50 isolates with a resistant phenotype (Dobner et al. 1997). They concluded that analysis of *inhA* was of limited value in predicting isoniazid resistance. Mutations in *inhA* can confer low level resistance to isoniazid.

#### 1.11.2.2.c *kasA*

KasA is an intracellular target for isoniazid which encodes a  $\beta$ -ketoacyl-ACP synthase. Mutations have been found within this gene which confer low levels of resistance to isoniazid (Zhang & Telenti 2000). However its role in isoniazid resistance is unclear as similar mutations have been identified in drug susceptible as well as resistant isolates (Lee et al. 1999; Piatek et al. 2000). Like *inhA*, it is involved in mycolic acid synthesis, to be more specific it is involved in elongation of fatty acids intermediate in the biosynthetic pathway of mycolic acids.

#### 1.11.2.2.d *ahpC*

Mutations in the promoter region of the gene *ahpC* have been found in approximately 10% of isoniazid resistant isolates but mutations in *katG* were also found in these isolates (Ramaswamy & Musser 1998; Sherman et al. 1996; Zhang & Telenti 2000). AhpC is homologous to other bacterial and eukaryotic proteins with alkylhydroperoxide reductase activities making it able to detoxify organic peroxides (Tartaglia, Storz, & Ames 1989). It has been suggested that mutations in *ahpC* promoters were a compensatory mechanism for the loss of *katG* activity in isoniazid resistant strains (Sherman et al. 1996). Further evidence that this is the case is the high incidence of strains with *ahpC* mutations which also carry *katG* mutations (Dhandayuthapani et al. 1996; Sherman et al. 1996). The changes within *ahpC* promoter regions are rare and not obligatory in isoniazid resistant organisms as strains with a KatG null phenotype are uncommon (Musser et al. 1996). Niimura et al discovered that it was likely that hydrogen peroxide was a substrate for *ahpC*



(Niimura, Poole, & Massey 1995). It is possible that AhpC may detoxify reactive oxygen intermediates formed during isoniazid activation.

In *E. coli* *ahpC* is regulated by *oxyR* which is a well characterised central regulator of peroxide stress response in enteric bacteria (Christman et al. 1985). However *M. tuberculosis* is a natural mutant of *oxyR* containing multiple mutations, making it a pseudo gene (Deretic et al. 1995; Deretic et al. 1996; Sherman et al. 1995). This is surprising because a key mechanism to ensure survival of *M. tuberculosis* within the macrophage is its ability to cope with oxidative stress response initiated by the human immune response. The loss of *oxyR* in *M. tuberculosis* may result in the high sensitivity of this organism to isoniazid (Deretic et al. 1995). The mutations in *oxyR* are present in all members of the *Mycobacterium* complex (Deretic et al. 1995). *M. smegmatis* is insensitive to isoniazid and has been found to produce high levels of AhpC (Dhandayuthapani et al. 1996) which indicates that *ahpC* expression in *M. tuberculosis* is defective, which can be attributed to the *oxyR* lesions in the tubercle bacillus (Deretic et al. 1995). Other evidence that *M. tuberculosis* does not express the *ahpC* gene has been proposed (Deretic et al. 1995; Dhandayuthapani et al. 1996). Dhandayuthapani et al demonstrated levels of *ahpC* expression in *M. tuberculosis* H37Rv below detection levels; leading to the interpretation that expression of this gene is inversely correlated to isoniazid sensitivity in mycobacterial species which were tested (Dhandayuthapani et al. 1996). *ahpC* upregulation has not been observed in *M. tuberculosis* isolates with *katG* mutations at codon 315 which reportedly lead to high MIC's against isoniazid and more than 20 fold decrease in KatG activity (Heym et al. 1995; Rouse et al. 1996). Analysis of the *oxyR-ahpC* region showed that mutations which lead to *ahpC* upregulation occurred at low frequencies and were

primarily localised in the intervening region between the two genes (Sreevatsan et al. 1997b). Also the mutations were predominantly restricted to isoniazid resistant isolates but did not always result in an upregulation of *ahpC*. Sreevatsan suggests that selection of *ahpC* upregulatory mutations are subjected to selective pressure by residual catalase-peroxidase activity among isolates with mutant *katG* (Sreevatsan et al. 1997b). The rarity and inconsistency of *ahpC* upregulation among isoniazid resistant isolates and *katG* mutant isolates indicates a complex relationship between the two and maybe due to the rare occurrence of complete *katG* deletion among *M. tuberculosis* isolates (Altamirano et al. 1994; Jaber, Rattan, & Kumar 1996; Pretorius et al. 1995).

### 1.11.3 Pyrazinamide

#### 1.11.3.1 Activity of Pyrazinamide

Pyrazinamide is a prodrug of pyrazinoic acid that is active only against *M. tuberculosis*, with scant or no effect on other mycobacteria (Konno, Feldmann, & McDermott 1967). It is an important first line drug for chemotherapy used in combination with isoniazid and rifampicin. It has been suggested that the target for pyrazinamide is the eukaryotic-like fatty acid synthetase I (FASI) of *M. tuberculosis* which is involved in fatty acid synthesis (Zimhony et al. 2000). Pyrazinamide requires amide hydrolysis by a pyrazinamidase to pyrazinoic acid by the mycobacterial enzyme pyrazinamidase (Konno, Feldmann, & McDermott 1967; Scorpio & Zhang 1996). It has been suggested that pyrazinamide diffuses passively into *M. tuberculosis* and once it is converted it accumulates intracellularly in its active form,

pyrazinoic acid, when the extracellular pH is acidic (Somoskovi, Parsons, & Salfinger 2001). This large accumulation occurs in the bacterial cytoplasm because of an inefficient efflux system (Salfinger, Crowle, & Reller 1990; Zhang et al. 1999) and lowers the intracellular pH which can reach sub-optimal levels. The lowering of the pH can result in the inactivation of the vital target enzyme FASII (Zimhony et al. 2000).

Pyrazinamide is a useful drug for combination therapy as it shortens the length of treatment which is highly desirable, from 1 year to 6 months when in combination with rifampicin (Grosset 1978). This is because of its excellent sterilizing ability at the start of treatment.

#### 1.11.3.2 Pyrazinamide Resistance

Resistance to pyrazinamide is due to mutations in the *pncA* gene that abolishes the amidase activity (from a loss of pyrazinamidase structural gene or missense mutations resulting in an altered allele). However, strains highly resistant to this agent do not always lack pyrazinamidase activity suggesting that a different resistance mechanism may also exist (Butler & Kilburn 1983; Davies et al. 2000b). These strains may not contain a *pncA* mutation so perhaps mechanisms involving pyrazinamide uptake, *pncA* regulation or pyrazinoic acid efflux may be involved in development of resistance (Raynaud et al. 1999). Scorpio & Zhang cloned and characterised the *M. tuberculosis pncA* gene and further sequenced pyrazinamide-resistant strains to demonstrate mutations in *pncA* (Scorpio & Zhang 1996). Pyrazinamide kills semi-dormant bacilli under acidic conditions (Heifets & Lindholm-Levy 1992; McDermott

& Tompsett 1954). *M. tuberculosis* encounters this low pH in intracellular compartments such as the macrophage phagolysosomes. However because of the difficulty of growing *M. tuberculosis* at pH 5.5, conventional susceptibility testing for pyrazinamide is problematic (McClatchy, Tsang, & Cernich 1981). Molecular based methods characterising *pncA* provide a better option. Approximately 70% of pyrazinamide resistant clinical isolates of *M. tuberculosis* have mutations in their *pncA* (Scorpio et al. 1997). Mutations in *pncA* in pyrazinamide resistant organisms have been found scattered throughout the gene (Scorpio & Zhang 1996) and include nucleotide deletions and insertions, missense alterations and termination mutations.

#### **1.11.4 Fluoroquinolones**

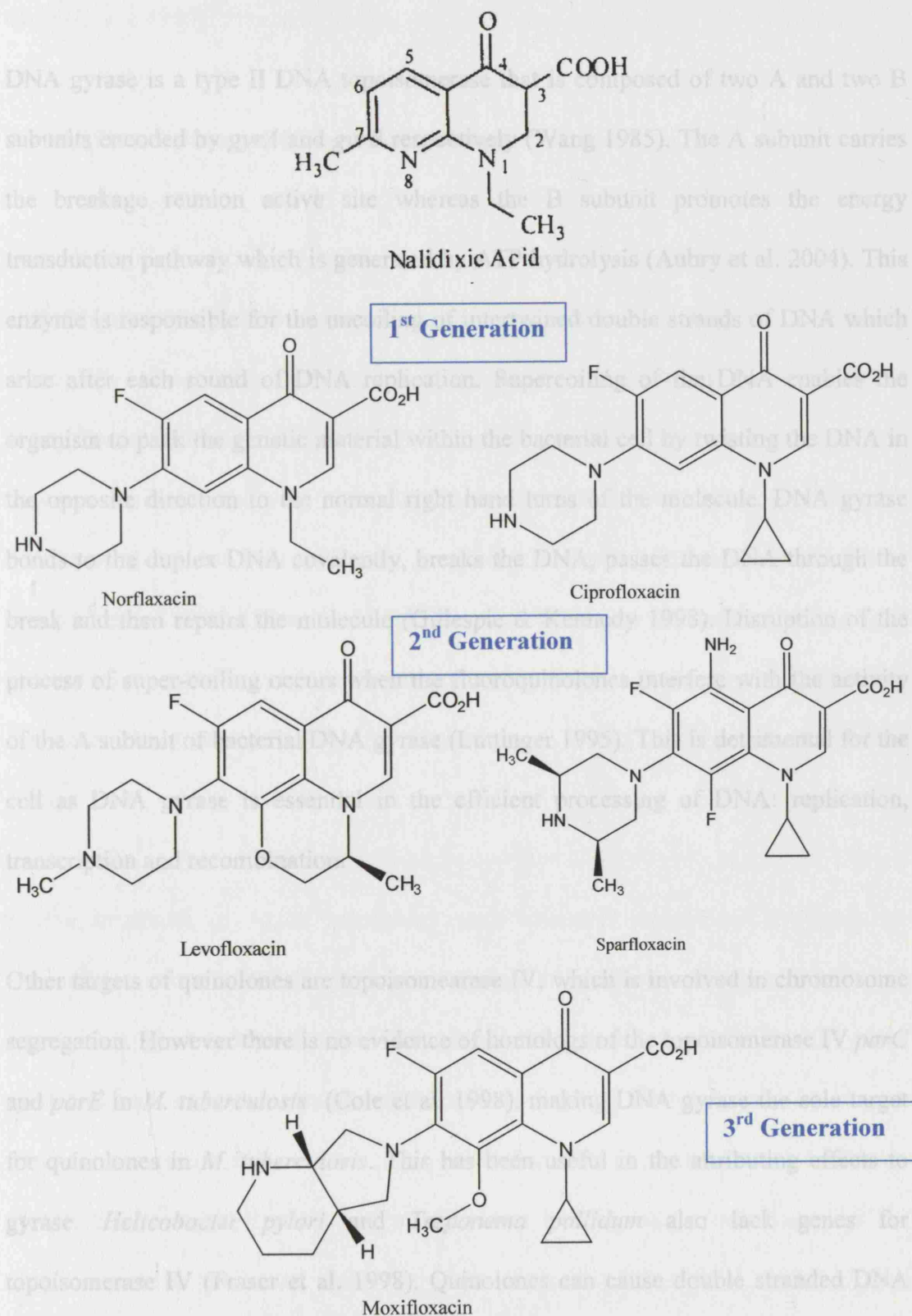
##### **1.11.4.1 Generations of Fluoroquinolone**

The fluoroquinolones are synthetic antibiotic structures that have bactericidal activity by targeting the enzyme DNA gyrase (figure 1.8). Ciprofloxacin, ofloxacin, levofloxacin, sparfloxacin, gatifloxacin and moxifloxacin are the most active 4-fluoroquinolones against *M. tuberculosis* (Berning 2001; Heifets 1991; Lounis et al. 2001). Fluoroquinolones are used largely in the treatment of MDR-TB when the patient cannot tolerate the regimen of rifampicin and isoniazid (Crofton, Choculet, & Maher 1997). These antimicrobial agents contain a 4-quinolone ring in their structure. There are many derivatives of quinolones with the prototype being nalidixic acid (figure 1.8). Nalidixic acid is non-fluorinated and active only against enterobacteria. In the 1980s the fluoroquinolones, a new generation of quinolone, were introduced. The newer fluoroquinolones, moxifloxacin, sparfloxacin and gatifloxacin, have lower

MIC's than levofloxacin, ciprofloxacin and ofloxacin *in vitro* against *M. tuberculosis* (Fung-Tomc et al. 2000; Ji et al. 1995; Ji et al. 1998; Tomioka et al. 1999; Truffot-Pernot, Ji, & Grosset 1991). Ciprofloxacin has an added fluorine at the C-6 position, an N-1 cyclopropyl group, a piperazinyl ring to the C-7 position. Moxifloxacin has a methoxy group attached to the C-8 position (Drlica & Malik 2003). It has been shown that substituents at this C-8 position increase activity particularly against resistant mutants. Dong et al showed that the C-8 methoxyl group improved bacteriostatic action against *M. tuberculosis* particularly against moderately resistant *gyrA* mutants when compared with C-8 hydrogen group (Dong et al. 1998). Moxifloxacin is the most active fluoroquinolone against *M. tuberculosis* when compared to ciprofloxacin, levofloxacin and sparfloxacin (Gillespie & Billington 1999) and is being evaluated as an effective first line treatment for pulmonary tuberculosis to replace other agents (Gosling et al. 2003). Fluoroquinolones are both bactericidal and bacteriostatic (Drlica & Zhao 1997) and are mildly mutagenic (Phillips et al. 1987; Power & Phillips 1993). One suggestion is that they induce mutations through the generation of free radicals (Arriaga-Alba et al. 2000). They are broad spectrum antibacterial agents that can display non-target based as well as target based resistance (Nakamura et al. 1994).

*In vivo* fluoroquinolones are readily absorbed after oral administration in humans and have good tissue penetration and distribution into the lungs and alveolar macrophages (Hooper & Wolfson 1985; Lubasch et al. 2000; Siefert et al. 1999). Fluoroquinolones have been shown to penetrate and exert bactericidal activity inside human macrophages (Rastogi, Labrousse, & Goh 1996).

Their target in *M. tuberculosis* is the essential bacterial enzyme DNA gyrase which controls the supercoiling of the bacterial DNA and relaxes any topological stress exerted on the DNA from chromosome replication and transcription (Onodera, Tanaka, & Sato 2001).

**Figure 1.9:** The structures of the generations of quinolone development

#### 1.11.4.2 DNA Gyrase

DNA gyrase is a type II DNA topoisomerase that is composed of two A and two B subunits encoded by *gyrA* and *gyrB* respectively (Wang 1985). The A subunit carries the breakage reunion active site whereas the B subunit promotes the energy transduction pathway which is generated by ATP hydrolysis (Aubry et al. 2004). This enzyme is responsible for the uncoiling of intertwined double strands of DNA which arise after each round of DNA replication. Supercoiling of the DNA enables the organism to pack the genetic material within the bacterial cell by twisting the DNA in the opposite direction to the normal right hand turns of the molecule. DNA gyrase bonds to the duplex DNA covalently, breaks the DNA, passes the DNA through the break and then repairs the molecule (Gillespie & Kennedy 1998). Disruption of the process of super-coiling occurs when the fluoroquinolones interfere with the activity of the A subunit of bacterial DNA gyrase (Luttinger 1995). This is detrimental for the cell as DNA gyrase is essential in the efficient processing of DNA: replication, transcription and recombination.

Other targets of quinolones are topoisomerase IV, which is involved in chromosome segregation. However there is no evidence of homologs of the topoisomerase IV *parC* and *parE* in *M. tuberculosis* (Cole et al. 1998), making DNA gyrase the sole target for quinolones in *M. tuberculosis*. This has been useful in the attributing effects to gyrase. *Helicobacter pylori* and *Treponema pallidum* also lack genes for topoisomerase IV (Fraser et al. 1998). Quinolones can cause double stranded DNA breaks which are lethal for the cell. These breaks are released from



quinolone/gyrase/DNA complexes which block DNA synthesis through replication forks (Drlica 1999).

#### 1.11.4.3 Development of Quinolone Resistance

Quinolones are increasingly being used against a variety of bacterial infections and with this clinical resistance has been emerging quickly in some bacteria, particularly among *Ps. aeruginosa* and methicillin resistant *Staphylococcus aureus* (MRSA) (Fung-Tomc, Kolek, & Bonner 1993). It has been indicated that in these organisms exposure to sub-inhibitory concentrations of ciprofloxacin can promote the development of low-level resistance to antibiotics with different modes of action (Fung-Tomc, Kolek, & Bonner 1993). The cross resistance between quinolones and non-quinolone anti-bacterials has been exposed in Gram negative bacteria (Celesk & Robillard 1989; Gutmann et al. 1985; Hooper et al. 1986; Piddock, Hall, & Walters 1991).

In the treatment of lower respiratory tract infection caused by *Streptococcus pneumoniae*, resistance of this pathogen to quinolone antibiotics has emerged through mutations in *gyrA* and *parC*. It has been shown these mutations predispose the genes *gyrA* and *parC* to further mutation (Gillespie et al. 2003). This is indeed a worrying trend. However *in vitro* studies have indicated that exposure to sub-inhibitory concentrations of gatifloxacin and moxifloxacin rapidly induce mutations and the mutation rate is strain and quinolone dose dependent (Eick et al. 2004). Komp Lindgren et al reported that an increased general mutation rate played a significant role in the development of high level resistance to fluoroquinolones, in *E. coli* isolates

from urinary tract infections, by increasing the rate of accumulation of rare new mutations (Komp Lindgren, Karlsson, & Hughes 2003).

It has been shown that exposure to sub-inhibitory concentrations of quinolone can increase the mutation rate of mycobacteria (Gillespie et al. 2005). Using Drakes method of measuring mutation rate, bacteria were exposed to  $\frac{1}{2}$ ,  $\frac{1}{4}$  and  $\frac{1}{8}$  MIC of ciprofloxacin (Drake 1970). When mutants were selected on rifampicin, erythromycin and gentamicin, those mutants exposed to  $\frac{1}{2}$  MIC ciprofloxacin had a 73-120 fold increase in mutation rate (table 1.2). There was a 120 fold increase in the mutation rate to moxifloxacin. This effect was found to be dose dependent as exposure to  $\frac{1}{4}$  MIC ciprofloxacin resulted in a low mutation rate and then exposure to  $\frac{1}{8}$  MIC an even lower rate. When using exposure to  $\frac{1}{2}$  MIC rifampicin as a control, the same effect was not observed suggesting it is not a sub-lethal dose phenomenon. The increase in mutation rate was found to all selecting agents which suggests that fluoroquinolones affect the whole genome since it became resistant to all the antibiotics tested so mutations must have occurred at several different genes (Gillespie 2002). Our evidence from *M. fortuitum* corresponds to previous data which shows that sub-inhibitory doses of fluoroquinolones result in an increased mutation rate to develop antibiotic resistance in *E. coli*, MRSA and *Ps. aeruginosa* (Drlica & Zhao 1997; Fung-Tomc, Kolek, & Bonner 1993; Phillips et al. 1987)

**Table 1.2:** Effect on the mutation rate of *M. fortuitum* (RF 01:332) when grown in the presence of sub-inhibitory levels of ciprofloxacin. Ratio (mean and standard error of mean for 5 median mutation experiments, 5 replicates/experiment) between the mutation rate for *M. fortuitum* grown with and without ciprofloxacin in the broth for six antibiotics; this ratio is an index of mutation rate. Table from Gillespie et al 2005.

Selective Agent	Concentration of ciprofloxacin in test broths			p ANOVA
	½ MIC (S.E.M.)	¼ MIC (S.E.M.)	⅛ MIC (S.E.M.)	
Ciprofloxacin	88.8 (36.6)	5.0 (1.2)	3.8 (0.6)	0.02
Levofloxacin	94.9 (35.7)	5.2 (1.3)	3.1 (0.5)	0.01
Moxifloxacin	121.1 (32.9)	5.6 (1.1)	3.9 (0.8)	0.0006
Rifampicin	81.7 (36.9)	21.2 (11.6)	4.2 (1.8)	0.003
Erythromycin	72.1 (29.4)	21.8 (10.8)	9.6 (4.9)	0.04
Gentamicin	102.5 (41.6)	29.7 (15.3)	6.8 (3.3)	0.007
<b>Control experiment, growth in ½ MIC rifampicin</b>				
Rifampicin	1.8 (mean of two median mutation experiments, 10 replicates per experiment)			

Sub-inhibitory concentrations of nalidixic acid were shown to be non-mutagenic in previous studies (Cook, Deitz, & Goss 1966). Nalidixic acid possesses only bactericidal activity against actively dividing cells (mechanism A) whereas the newer fluoroquinolones possess additional mechanisms; against non-dividing cells (mechanism B and C) (Howard, Pinney, & Smith 1993). The bactericidal activity of quinolones has been characterised by their mechanism of action in phosphate-buffered saline (PBS). Mechanism A requires RNA and protein synthesis and this activity is shared by all quinolones (Lewin, Morrissey, & Smith 1991). Mechanism B does not

require multiplying bacteria or RNA and protein synthesis and mechanism C does not require multiplying bacteria but needs RNA and protein synthesis (Gradelski et al. 2002; Lewin, Amyes, & Smith 1989). It has been shown that DNA damage that results from bacterial mechanisms B and C is subject to SOS mutagenic repair unlike from bacterial mechanism A (Howard, Pinney, & Smith 1993). It has been suggested that quinolones are less mutagenic in cells which cannot mount a SOS response; stationary-phase cells, RecA<sup>-</sup> mutants (Levin, Marnett, & Ames 1984) and *gyrA* mutants (Phillips et al. 1987).

#### 1.11.4.4 Quinolone Resistance due to *gyrA* and *gyrB* Mutations

It is well known that resistance to fluoroquinolones in *M. tuberculosis* arises readily (Rastogi et al. 1992; Sullivan et al. 1995). The resistance allele selected, *gyrA* or *gyrB*, is dependent on fluoroquinolone concentration. Most mutations conferring low level resistance to quinolones contain variants of the *M. tuberculosis* GyrB and at higher quinolone concentrations, GyrA variants are recovered (Zhou et al. 2000). Most mutations recovered in clinical isolates of *M. tuberculosis* occur primarily at GyrA codon 94, and also at positions 88 and 90 with occasional isolation of a *gyrB* mutant (Lubasch et al. 2001; Perlman et al. 1997; Sullivan et al. 1995; Takiff et al. 1994; Xu et al. 1996). A study using the laboratory strain *M. tuberculosis* H37Ra revealed that mutations in *gyrB* were only present as a secondary mutation to *gyrA* (Kocagoz et al. 1996). The cloning and sequencing of *gyrA* and *gyrB* has identified mutations, as in other bacteria, clustered in a small region in *gyrA* which is approximately 40 residues amino-terminal to the catalytic tyrosine, Tyr122 in *E. coli*, involved in DNA strand scission (Reece & Maxwell 1991; Takiff et al. 1994). Single amino acid substitutions

have also been identified in *M. tuberculosis* clinical isolates at residues 88 to 94 in conferring resistance to ciprofloxacin. Codons 89, 90, 91, 94 and 95 in *gyrA* have been shown to be polymorphic (Ramaswamy & Musser 1998; Takiff et al. 1994; Xu et al. 1996). The Ser95Thr substitution has been identified in resistant and susceptible isolates so this substitution is apparently unrelated to ciprofloxacin resistance (Kapur et al. 1995).

*M. tuberculosis* is unusual in that it has an alanine at position 83 in GyrA (*E. coli* numbering), where the equivalent residue is serine (Barnard & Maxwell 2001). This difference may be of key importance for quinolone interaction. The activity of quinolones differs between Gram negative and Gram positive bacteria (Hooper 2002). In Gram negative organisms, gyrase is more sensitive to the effects of fluoroquinolone attack than topoisomerase IV, therefore primary mutations conferring resistance are observed in *gyrA*, rather than in *parC* and *parE* (Blanche et al. 1996; Breines et al. 1997; Chen et al. 1996; Khodursky, Zechiedrich, & Cozzarelli 1995). In *S. aureus*, representative of Gram positive bacteria, gyrase is less sensitive to fluoroquinolones than in Gram negative bacteria, with sensitivity to topoisomerase IV being roughly the same in both types (Drlica & Hooper 2003). *M. tuberculosis* does not follow this pattern. A Ser83Ala change in *E. coli* GyrA confers resistance to quinolones, even though it has been shown to have no effect on the DNA-supercoiling activity of gyrase (Barnard & Maxwell 2001; Hallett & Maxwell 1991).

#### 1.11.4.5 Quinolone Resistance due to Other Mechanisms

Quinolones must cross the cell wall and cytoplasmic membrane of mycobacteria to reach DNA gyrase present in the cytoplasm. Mechanisms of resistance, not involving mutations in *gyrA*, exist including changes in cell wall permeability or active quinolone efflux pumping (Sullivan et al. 1995). In *M. smegmatis* a possible permeation mechanism is predicted to encode an efflux pump, the *lfrA* gene. Homologs of *lfrA* appear to be present in *M. tuberculosis* (Takiff et al. 1996). Overexpression of a clone of this gene from a plasmid in *M. smegmatis* has been shown to be associated with resistance to quinolones (Liu, Takiff, & Nikaido 1996). This has yet to be demonstrated in chromosomal *lfrA* in *M. smegmatis*. The protein does appear to contribute to the innate reduced susceptibility of mycobacteria to quinolones (Sander et al. 2000).

#### 1.11.4.6 Induction of the SOS Response by Fluoroquinolones

Quinolones were not thought to be mutagenic, when examined by conventional mutagenicity assays (Ames test, V79 cell line; the test for induction of chromosome aberrations in V79 cells, DLT; cominant lethal test, and MNT; micronucleus test) but it is clear that they induce the SOS response (Phillips et al. 1987; Piddock & Wise 1987). Fluoroquinolones have however been shown in other studies to be mutagenic (Mamber et al. 1993). They disrupt the action of DNA gyrase which results in double stranded DNA breaks (Drlica & Zhao 1997). This results in potent induction of the SOS regulon, a set of genes involved in DNA repair, recombination and mutagenesis (Gudas & Pardee 1975; Phillips et al. 1987; Walker 1984). The first studies of this were

done in *E. coli* where it was shown that nalidixic acid induced the SOS response (Casaregola, D'Ari, & Huisman 1982; Gudas & Pardee 1976; Little & Mount 1982). There is strong evidence which shows that regions of exposed single-stranded DNA in the cell are generated. However subsequent processing of the DNA by RecBCD nuclease is required to unwind the DNA from double strand breaks for nalixic acid to function as an SOS inducer (Chaudhury & Smith 1985; Gudas & Pardee 1975; Karu & Belk 1982). The mechanism for induction of low-level resistance is thought to be as a result of quinolone induction of the SOS response (Mamber et al. 1993; Power & Phillips 1992). Phillips et al showed an induction of the SOS response in *E. coli* exposed to 4-quinolones; ciprofloxacin, olaquinox, difloxacin, enoxacin, norfloxacin and ofloxacin (Phillips et al. 1987). They further showed maximum induction of the SOS processes at the higher quinolone concentrations (about 10 fold the MIC). The correlation between SOS error prone repair and mutagenic effects indicate that quinolone-induced mutagenic effects in bacteria are almost entirely due to SOS-processed DNA damage (Power & Phillips 1993). Therefore quinolone therapy could lead to an increase in the development of antibiotic resistance. This means that caution should be used when adding a fluoroquinolones to the treatment regimen for *M. tuberculosis* and other respiratory tract infections, as cross-resistance to other antibacterials may develop. Mycobacteria in different compartments of the body are in different physiological states (Gillespie & Kennedy 1998; Ginsburg, Grosset, & Bishai 2003) and it is thought that each population has varying susceptibilities to anti-tubercular drugs (section 1.10.1). So the regimen prescribed must be chosen to minimize the risk of exposing bacteria to sub-inhibitory concentrations of fluoroquinolone.

Induced mutagenesis can also result in enhanced survival of quinolone resistant mutations in the presence of fluoroquinolones (Bisognano et al. 2004). However members with C-8 position substituents increase their efficacy at killing first step gyrase and topoisomerase IV resistance mutants (Dong et al. 1998; Ito, Matsumoto, & Nishino 1995; Zhao et al. 1997; Zhao et al. 1998). This is important as instead of just blocking growth which may allow more mutations to occur, cells are killed, including any induced mutants. Otherwise quinolone treatment may lead to the formation of resistant cells.

#### 1.11.5 Streptomycin

Streptomycin was the first antibiotic to be used as an effective antitubercular agent in 1944 (Schatz & Waksman 1944). It was purified from *Streptomyces griseus*. It proved to be useful in killing susceptible populations of bacteria but alone it was not sufficient to cure most cases, leaving behind mutant progeny. The activity of this antibacterial, among other aminoglycosides, inhibits initiation of mRNA translation, along with affecting translational accuracy (Cundliffe 1981; Ruusala & Kurland 1984). The rate at which *M. tuberculosis* develops resistance to drugs was found highest for streptomycin and lowest for rifampicin (Coninx et al. 1999; Davies et al. 2000a). Mutations associated with streptomycin resistance have been identified in two targets, the 16S rRNA gene (*rrs*) and the gene (*rpsL*) encoding ribosomal protein S12 (Douglass & Steyn 1993; Finken et al. 1993). Mutations in *rpsL* are mainly associated with high level resistance, mutations in *rrs* are associated with intermediate level resistance, and wild type *rpsL* and *rrs* exhibited low level streptomycin resistance (Meier et al. 1996). An interesting observation is that *M. tuberculosis* like other slow



growing mycobacteria only has one copy of the 16S rRNA so that single nucleotide changes can result in antibiotic resistance (dominant behaviour). Approximately 1/3 of streptomycin resistant clinical isolates lack changes in the *rrs* or *rpsL* genes (Finken et al. 1993; Heym et al. 1994), indicating that there are alternate mechanisms of resistance such as a permeability barrier (Shaila, Gopinathan, & Ramakrishnan 1973). Meier et al investigated this alternative resistance mechanism using a membrane active agent (Tween 80) which they discovered may confer low levels of resistance to streptomycin (Meier et al. 1996). However this remains to be confirmed. The S12 protein is well conserved among streptomycin resistant mycobacteria such as *M. avium*, *M. goodii* and *M. szulgai* which further suggests that permeability barriers may contribute to resistance (Honore & Cole 1994; Meier et al. 1996).

#### 1.11.6 Ethambutol

The primary site of action of ethambutol is arabinan biosynthesis. Arabinogalactan is a polysaccharide which is a component of the acid fast cell wall of *Mycobacterium*. In *M. smegmatis*, ethambutol inhibits the transfer of arabinogalactan to the cell wall which is proposed leads to the accumulation of mycolic acids (Kilburn et al. 1981). Preliminary studies by Takayama et al have demonstrated inhibition of transfer of mycolic acids into the cell wall after 15 min of drug administration in *M. smegmatis* (Takayama et al. 1979). The genes *emb A*, *B* and *C* encoding putative ethambutol targets are arranged as a 10kb operon in *M. tuberculosis* (Telenti et al. 1997). Studies of ethambutol resistant clinical isolates have revealed the occurrence of missense changes in the *embB* codon 306 (Mokrousov et al. 2002; Telenti et al. 1997). It is likely that mutations at codon 306 are not just a marker for ethambutol resistance but

actually mediate resistance (Sreevatsan et al. 1997c). About 30% of ethambutol resistant clinical isolates have no *embB* mutation (Ramaswamy & Musser 1998) which suggests alternative mechanisms of resistance which remain to be discovered.

## 1.12 Heteroresistance

### 1.12.1 The Study of Heteroresistance

The phenomenon of heteroresistance is the simultaneous occurrence of drug resistant and drug sensitive organisms in a single clinical specimen or isolate prior to drug exposure. The proportion of resistant organisms is in excess than would be predicted by the spontaneous mutation rate and cannot be explained by the natural 'background' rate of mutation. Heteroresistance has been most studied in *S. aureus* (Hiramatsu et al. 1997; Ryffel et al. 1994), specifically to vancomycin in MRSA. The precise genetic determinants for vancomycin resistance in staphylococci remain to be elucidated (Van Der Zwet et al. 2002). It is understood in these species that methicillin resistance is as a result of a mutation in the structural gene *mecA* and whether this is the causative mechanism of heteroresistance or just a pre-requisite is still questionable. The structural gene *mecA* encodes for a low affinity penicillin binding protein (PBP2') which when expressed confers basal line resistance to  $\beta$ -lactams. In an editorial, Rohrer et al posed the idea that autolysins were involved in making resistance to methicillin heterogeneous (Rohrer, Maki, & Berger-Bachi 2003). Autolysins are involved in cell death and cell separation in the presence of penicillin; thereby alterations in their activity can also influence the level of resistance in MRSA. Alam et al published findings of the first report of hetero-vancomycin-resistant *Enterococcus faecium* isolate from a patient (Alam et al. 2001). Initial isolates from the patient were susceptible and subsequent isolates were hetero-vancomycin-resistant. Reports on heteroresistance have also been described in *Gardnerella vaginalis* to the drug metronidazole (Altrichter & Heizmann 1994) and in two patients with *Cryptococcus neoformans* infection to fluconazole and voriconazole (Mondon et

al. 1999). The resistance in *C. neoformans* was innate and unrelated to drug exposure as one of the patients had never been treated with azoles. A heteroresistant phenotype was shown in a *Staphylococcus capitis* strain in a neonatal intensive care unit (Van Der Zwet et al. 2002). Two hundred and seventeen *S. capitis* strains were screened of which heteroresistance to vanomycin was found in 48 strains. In a study of *H. pylori* isolates from patients, 41 out of 220 (19%) were infected with heteroresistant bacteria to at least one of the antibiotics tested, with the phenomenon most common among metronidazole-resistant *H. pylori* (28 cases) (Kim, Kim, & Kwon 2003). This study tested whether heteroresistance was as a result of co-infection with a second strain using genotype analysis and found that resistant *H. pylori* was more likely to develop from pre-existing susceptible strains by genomic alteration rather than co-infection. However it is possible that genomic material from a resistant strain can transform a susceptible strain.

### **1.12.2 Causes of Heteroresistance in Mycobacteria**

#### **1.12.2.1 Antibiotic Penetrating Ability**

As mentioned it has been hypothesised that mycobacteria can exist as discreet populations within the patient: in the bronchioles, alveoli, cavities, macrophages and in caseous foci (Mitchison 1979). Each population has a different susceptibility to the anti-tubercular agents which will determine the efficacy of the agent (Yamori et al. 1992). If at each of these sites in the body there are also mixed populations of drug sensitive and drug resistant bacilli, as has been suggested, this creates further problems in that a drug will target only the sensitive population. If only a single agent

is effective in a 'compartment' (e.g. pyrazinamide in the acidic environment in caseous foci) and a heteroresistant population of bacteria exists, a selection of a singly resistant mutant could occur. This may suggest that heterogeneity in the exposure of the mycobacteria to different antibiotic agents due to differences in penetration or bacterial killing may be a mechanism of emergence of resistance (Lipsitch & Levin 1998).

#### **1.12.2.2 Inadequate Therapy**

In the absence of protected compartments it is suggested that resistance occurs due to non-compliance with treatment rather than heterogeneity of drug exposure (Lipsitch & Levin 1998). The presence of a large compartmentalised bacillary load, inadequate drug therapy or the level of heteroresistance in a *M. tuberculosis* strain may lead to an increase in the risk of progression to homogenous phenotypic resistance. At present it appears that the greatest risk factor for the development of resistant tuberculosis is inadequate therapy.

#### **1.12.2.3 Growth Cycle of Bacteria**

Spontaneous mutation rates vary depending on the phase of the growth cycle of the bacteria. It is possible that a heteroresistant strain is generated by a large number of bacteria being in post exponential growth phase. There are numerous studies showing growth advantage of mutants in stationary phase, in which sub-populations of mutant cells take over stationary phase cultures (Zambrano & Kolter 1996). An increase in mutation rate up to  $10^4$  fold has been shown in this phase in *M. smegmatis*

(Karunakaran & Davies 2000). It may be possible that a sigma factor, known to regulate expression of many genes in stationary phase, associated down regulation could explain the occurrence of hypermutation in cultures which enter late stationary phase. The activation of error prone DNA polymerases at the end of stationary phase and in starvation may explain the increase in mutation (Layton & Foster 2003).

#### **1.12.2.4 Defective DNA Repair System**

Also within a bacterial population, sub-populations exist with an increased rate of mutation due to a reduction or a defect in the expression of proteins involved in DNA repair. Generation of multiple alleles with different resistance phenotypes can occur with subsequent maintenance of a mutator sub-population multiplying in stressed conditions (Martinez & Baquero 2000).

#### **1.12.3 Detection of Heteroresistance**

Heteroresistance has been shown to compromise the detection of mutation in clinical practice thereby influencing the sensitivity of molecular diagnostic tests for the detection of resistance such as PCR followed by direct sequencing or single strand confirmation polymorphism (Heep et al. 2001). This problem may be resolved by performing multiple PCR product characterisations from multiple PCR's of the same target. This strategy would prove very costly and the problem may be resolved by using a PCR-based line probe assay (INNO-LiPA) or RFLP, but these steps all still rely on DNA amplification. This can result in a mixed PCR product with sensitive and resistant populations.

One study found mixed populations of wild type and mutation patterns with INNO-LiPA (Marttila et al. 1999). This is a reverse hybridization-based assay where colonies with nucleotide changes, which encode resistance, will impede the binding of the target and corresponding wild-type probes (Rossau et al. 1997). Heep et al demonstrated the presence of mixed subpopulations of *M. tuberculosis* to rifampicin, of which they found heteroresistance in 8 out of 80 clinical *M. tuberculosis* strains isolated in Germany during 1997 (Heep et al. 2001). However, the detection of resistant mutations by direct sequencing of PCR products was inhibited due to the presence of sensitive and resistant sub-populations in the initial bacterial culture. So instead, specific probes could be used to detect mutations in small populations.

Streptomycin sensitive and resistant colonies have been detected in large broth cultures of *M. tuberculosis* H37Rv (Vennesland, Ebert, & Block 1947). The presence of resistant variants in this experiment is likely to be clinically relevant. They must be as a result of spontaneous mutation which is likely to occur in a previously untreated patient rather than selection. Mixed populations in an isolate from a treated patient reflect inadequate treatment. Genotypic heteroresistance was detected to ethambutol in sputum samples from patients in Tanzania infected with pulmonary tuberculosis (Cullen et al. 2006). The phenomenon was detected in 3/6 samples (2 out of 3 patients) by the presence of mixed populations of wild-type and  $\Delta embB$  genes bearing a mutation at codon 306 of *embB*. These mutations occurred even though the risk of drug resistance was low in this Tanzanian population as a result of active participation in the DOTS programme (section 1.6.8). Rinder et al have shown the presence of ethambutol sensitive and resistant *M. tuberculosis* genotypes, by analyzing *embB*,

within the same clinical sample for patients who have a high probability of having resistant tuberculosis (Rinder, Mieskes, & Loscher 2001). They also showed the same phenomenon with isoniazid and streptomycin by analysing *katG* and *rpsL* (Rinder et al. 1999). From this they concluded that heteroresistance is not restricted to a particular resistance gene and it is not a rare phenomenon. It possibly affects all genes whose products are targeted by antibiotics.

The presence of mixed populations of bacteria prior to drug exposure could explain treatment failures not only for tuberculosis but also for other bacterial infections. The best method of detection of this phenomenon remains to be clarified as routine sensitivity testing is of no benefit. Individuals with tuberculosis would be expected to harbour only a very small number of drug resistant bacilli as predicted by the spontaneous mutation rate of the organism to each agent (at a rate of approximately 1 resistant mutant per  $10^7 - 10^9$  organisms). The progression to homogenous resistance is therefore not simply associated with spontaneous mutation. For heteroresistance to have occurred the prevalence of resistant organisms must be greater than the spontaneous mutation rate. It is difficult to detect small numbers of resistant bacteria by culture or even molecular methods. One possible approach may be to use specific hybridization to detect small subpopulations of resistant bacteria. Investigation into heteroresistance has been facilitated by the E-test; subpopulations of resistant bacteria appear in the clear zone of inhibition in the halo which forms around the strip (Alam et al. 2001; Mondon et al. 1999). However the use of E-tests can only be used as an indicator and requires further investigation, such as the plating of single colonies of the organism onto varying drug concentrations above and below the MIC.



#### 1.12.4 Clinical Significance

Heteroresistance is not a rare phenomenon (Rinder, Mieskes, & Loscher 2001) but phenotypically it is not observed in routine clinical tests, as it has been shown to confound molecular tests, so its clinical significance is not clear. There is evidence which suggests that heteroresistant isolates are more likely to convert to homogenous resistance than susceptible strains (Hiramatsu et al. 1997). The resistant population do not overwhelm the sensitive one in the absence of drug. This may be because the relative fitness of the resistant sub-population is decreased in relation to the sensitive population so the wild-type sensitive population outgrows the biologically less fit resistant organisms. It has been shown that a decrease in growth rate can occur as a result of an acquisition of resistance (Billington, McHugh, & Gillespie 1999; Gillespie 2001). This predicts that in the absence of antibiotic selective pressure, resistant populations will be outgrown by the pre-domination of sensitive organisms. The ability of the strain to overcome the fitness deficit through compensatory mutations may lead to the maintenance of low numbers of resistant bacteria within the same strain. It is important to determine whether heteroresistance occurs as a result of infection with two strains with different genetic profiles or whether the resistant population is clonal and develops during the course of infection.

#### 1.12.5 Genetic Mechanism of Heteroresistance

The genetic mechanism by which heteroresistance can occur in *M. tuberculosis* remains to be elucidated. It has been suggested that members of the Mut family of proteins may be involved (Rad et al. 2003). For instance *mutT2* is a strong specific

mutator and it has been shown to enhance A·T → C·G transversions in *E. coli* without any effect on other types of mutations (Fowler et al. 2003). MutT is a member of the GO system responsible for removing an oxidatively damaged form of guanine from DNA and the nucleotide pool (section 4.1.4). A possible mutation in this gene may result in an increased resistance to drugs due to a defect in DNA repair systems. It has been shown that some isolates of the Beijing family of *M. tuberculosis* contain a mutation in *mutT*, in addition to mutations in other DNA repair genes (Rad et al. 2003). The Beijing genotype is reported to correlate with drug resistance (Toungoussova et al. 2002).

### **1.13 Multi-Drug Resistant Tuberculosis (MDR-TB)**

#### **1.13.1 Epidemiology**

MDR-TB is defined as tuberculosis which is resistant to two or more anti-tuberculosis drugs including rifampicin and isoniazid. WHO is addressing the problem of drug resistance in combination with several partners around the world. For instance in 1994, they launched a global survey to investigate the incidence of resistance with first-line anti-tuberculosis agents (Pablos-Mendez et al. 1998). This has raised some concern as MDR-TB was found in all 35 countries surveyed except Kenya. Thus, indicating a need to tackle MDR-TB as a priority issue in human health. MDR-TB poses a great threat to all countries due to the increased circulation of drug-resistant strains due to poor tuberculosis control. A further report in 2000 surveyed 72 countries and confirmed that MDR-TB was widespread and that it had occurred in Kenya where it had previously not been a problem (WHO/IUATLD 2000). It is estimated that there were 273,000 new cases of MDR-TB out of 8.7 million new cases in 2000 (3.2%) (Dye et al. 2002a). Out of 64 countries, the highest MDR proportions among new cases have been found in Estonia (14%), Henan Province in China (11%), Lativa (9%) and Ivanovo (9%) and Tomsk (7%) provinces in Russia (Espinal et al. 2001). It is a serious problem in Russia where drug resistance is associated with accelerated transmission rates and has posed a threat to existing progress in the treatment of tuberculosis (Perelman 2000). One third of MDR-TB worldwide comes from Russia (Orellana 2002). Resistance is very unevenly distributed around the world with an estimated 70% of new MDR-TB cases in just 10 countries (Dye et al. 2002b). Thus MDR cases are a local problem rather than a global one, although its

spread has been classed as a global pandemic (Farmer & Kim 1998). It has even been suggested by Nitta et al that there is limited transmission of MDR-TB (Nitta et al. 2002). Teixeira et al showed that when assessing rates of infection and progression of TB between household contacts of patients with MDR and drug susceptible pulmonary tuberculosis, MDR-TB was readily transmissible to children and associated with a high rate of infection and disease (Teixeira et al. 2001). Even though it has been suggested that MDR-TB patients are less infectious, MDR-TB is a chronic disease, and even if it is less infectious, lower transmissibility may be compensated for by a longer period of disease.

A study into MDR-TB in the UK in 2002 identified three factors which influence the survival time for MDR-TB patients: age, appropriate treatment and the immunological status of the patient (Drobniewski et al. 2002). This study showed a pattern of increasing risk with age. Patients who received appropriate three drug treatment have a longer median survival time and a lower chance of death. Immunocompromised patients had shorter median survival times; they were 9 times more likely to die than immunocompetent patients. Although incidence of MDR-TB is low in the UK, the proportion of initial multi-drug resistant isolates was 1.7% in 1996, an increase from 0.6% in 1993 (Irish et al. 1999).

### **1.13.2 Treatment of MDR-TB**

The treatment of cases resistant to rifampicin and isoniazid requires deviation from standard short course regimens with first line agents, which is associated with higher mortality rates, treatment failure rates and increased periods of transmission (WHO

2003). A mathematical model derived by Dye and Williams shows that standard therapy is not sufficient with a less than 60% cure rate once multi-drug resistance has developed (Dye & Williams 2000). There is a graded response which is dependent on the concentration of the drug administered, the number of drugs that the strain has developed resistance to, the immunocompetence of the patient and their compliance with treatment. In some studies short course chemotherapy is sufficient to prevent and reverse the spread of MDT-TB. Migliori et al found that the frequency of disease recurrence among MDR-TB patients declared 'cured' after short course chemotherapy is high (Migliori et al. 2002). They used culture based bacteriological confirmation at the end of treatment to ensure that the patient is cured. Although short course chemotherapy is successfully containing resistance in some parts of the world, there needs to be improved resistance testing and surveillance. With appropriate, intensive treatment regimens, most patients with multi-drug resistance can be treated (Tahaoglu et al. 2001).

For the treatment of multi drug resistant strains of tuberculosis, the regimen should begin with 5 or more drugs to which the organism is likely to be sensitive to and this treatment should continue until sputum cultures become negative. Then at least 3 drugs are administered for a minimum of 9 months and perhaps up until 24 months depending on the level of resistance *in vitro* of the drugs, the drugs available and the patient's HIV status. The second line antitubercular agents used are cycloserine, ofloxacin, ciprofloxacin, moxifloxacin, clarithromycin, azithromycin, kanamycin, amikacin, capreomycin, para-aminosalicylic acid or can be derivatives of first line drugs – rifabutin (rifampicin derivative), ethionamide (isoniazid). Moxifloxacin, a methoxyquinolone, has bactericidal activity against *M. tuberculosis* similar to that of

isoniazid *in vitro* and in mice (Gradelski et al. 2001). Ciprofloxacin has been shown to have significant early bactericidal effect, similar to isoniazid, against *M. tuberculosis* (Jindani et al. 1980). Fluoroquinolones should not be used alone or as first-line therapy and they should be reserved for the treatment of multi-drug resistant tuberculosis. Administration of a fluoroquinolone alongside pyrazinamide for 6 to 12 months may be considered as prophylaxis in areas with MDR-TB (Ghaffar & McCracken 2003).

The cost to treat MDR-TB is approximately 50 times more expensive than the cheapest short course regimen for drug susceptible patients (Gupta et al. 2001). Chronic MDR-TB patients can be treated with second-line drugs. This treatment may involve kanamycin (for the 1<sup>st</sup> 3 months), ciprofloxacin, ethionamide, pyrazinamide and ethambutol for 18 months. The average cost of this treatment per patient in the US is \$2,400 (Suarez et al. 2002). In the highest cost scenario, individualised treatment is assumed to cost \$10,000 per patient. Individualised treatment for some patients and earlier use of second-line drugs would increase cure rates which will never be as high as those for drug sensitive cases. It has been suggested that MDR-TB in low-income settings may be untreatable partly due to the high costs of treatment regimens (Iseman, Cohn, & Sbarbaro 1993; White & Moore-Gillon 2000).

To treat MDR-TB, WHO have conceived a DOTS-PLUS strategy which is involved in accessing expensive antitubercular, second line drugs in use in approved control programmes in middle income countries (WHO 2001). This strategy works as a supplement to DOTS.

### 1.13.3 Genetic Mechanisms of Multiple Drug Resistance

Multiple resistance occurs due to the stepwise acquisition of single independent mutations conveying resistance to different antibiotics. This is due to selective pressure for such resistant mutants exerted on the tubercle bacilli as a result of exposure to drugs. The sequence of mutations giving resistance to one drug or a group of drugs (e.g. rifamycins) occurs one at a time. In other pathogenic organisms, extra-chromosomal genetic elements (i.e. plasmids) can transfer resistance causing a rapid change from wild type susceptibility to multi-drug resistance to several unrelated antibacterial substances. This can occur in one single step, which is unlike in *M. tuberculosis* where no sudden development of multiple resistance can take place (Petrini & Hoffner 1999). The risk of developing resistance spontaneously under optimal treatment would be the combined mutation frequencies of *M. tuberculosis* to rifampicin ( $3.1 \times 10^{-8}$ ) and isoniazid ( $3.5 \times 10^{-6}$ ) which is  $9 \times 10^{-14}$  (Long 2000). Spontaneous development of multiple resistance is virtually impossible since there is no single gene involved in such a process and mutations resulting in resistance to various different classes of drugs are genetically unlinked (WHO 2003). Usually MDR-TB develops with isoniazid resistance first as this drug has been in use for the longest period of time and due to the fact that it has a high mutation rate, a hundred times higher than that leading to rifampicin resistance. Thus rifampicin resistance is understood to occur after isoniazid mono-resistance, leading to the stepwise development of MDR-TB.

Decreased membrane and cell wall permeability and the activation of drug efflux mechanisms are two common mechanisms in prokaryotes in the development of

resistance to multiple drugs and for limiting drug uptake (Nikaido 1994). Finken et al investigated this phenomenon in the development of streptomycin resistance in *M. tuberculosis* (Finken et al. 1993). They found that both single and multiple resistant isolates had the mutations in drug target genes occurring in the same positions so multiple resistance occurred by an accumulation of drug target specific mutational events. This eliminated the possibility that multiple resistance occurs due to a single alteration in a multiple resistance gene.



## **1.14 Mutation**

### **1.14.1 Mutagens**

All organisms undergo mutations, whether they are due to normal cellular functions, random interactions with their environment (spontaneous mutations) or due to interaction with mutagens. Most mutagens interact directly with the base pair sequence of DNA either by changing the base, deleting part of a sequence or inverting the remaining sequence (table 1.3). Single base pair mutations are termed point mutations and can lead to a single amino acid change in a protein or no change at all, depending on the codon involved. Inversions and deletions have a more profound effect on DNA causing frameshift mutations and can often result in loss of phenotype. Mutagens can be chemical or biological, can involve radiation and UV light (table 1.3). There are wide variations in the rate at which mutations can occur.

**Table 1.3:** The effect of mutagens on bacteria. Most mutagens result in the damage of DNA due to reactive oxygen species (OH radicals, superoxide anions and hydrogen peroxide). During metabolism of certain compounds, active oxygen species are also created and these result in the production of a variety of products (thymine glycols, 4,6 diamino-5 formamidopyrimidine, 2,6 diamino-4-hydroxy-5 formamidopyrimidine, 8 hydroxyguanine and strand breaks).

Mutagen	Effect on DNA
UV light	Pyrimidine dimers (thymine-thymine and thymine-cytosine dimers)
Hydrogen peroxide	DNA single strand breakage, damage to/loss of bases
Ionizing Radiation	breaks in one or both strands (can lead to rearrangements, deletions, chromosome loss, death if not repaired), damage to/loss of bases, crosslinking of DNA to itself or proteins
Nitric Oxide	pyrimidine excision inhibition
Ethidium bromide	Intercalates between base pairs
Iron (reduced form, FE (II))	DNA single strand breakage
Alkylating agents (e.g. MNNG, Dimethylnitrosamine)	Add methyl, ethyl and more complicated alkyl groups to nucleic acid bases

(MNNG = N-methyl-N'-nitro-N-nitrosoguanidine)

### 1.14.2 Oxidative Stress

#### 1.14.2.1 Causes of Oxidative Stress

Oxidation is perceived to be the most frequently encountered stress for *M. tuberculosis* within the human macrophage. To survive in this environment, the tubercle bacillus must protect itself against intracellular bactericidal mechanisms within the host mononuclear phagocyte, including the production of reactive oxygen intermediates which diffuse freely through the host cell (Gordon & Hart 1994; Russell, Dant, & Sturgill-Koszycki 1996). By-products of aerobic metabolism can lead to reactive oxygen molecules in the forms of hydrogen peroxide, hydroxyl radicals and superoxide. Superoxide is converted to hydrogen peroxide via superoxide dismutase, hydrogen peroxide is then converted to water via catalase. Oxidative stress can also be caused by environmental agents such as UV irradiation or redox-cycling agents which generate oxygen free radicals. This increase in active oxygen exceeds the level with which the cell can cope with, thereby stressing the cell. The nucleic acid and sugar moieties are susceptible to the oxygen free radicals produced, leading to base degradation, single strand breakage and cross linking to proteins. *In vitro* each of these reactive oxygen intermediates is bactericidal or bacteriastatic but their concentration in the human macrophage *in vivo* is still uncertain.

#### 1.14.2.2 Protective Mechanisms against Reactive Oxygen Intermediates

Within the host mononuclear phagocyte, *M. tuberculosis* resides in the phagosome which does not mature along the endocytic pathway (Russell, Dant, & Sturgill-

Koszycki 1996; Xu et al. 1994). Within the vacuole, the organism must protect itself against reactive oxygen and nitrogen intermediates which diffuse freely through the cell (Gordon & Hart 1994). The genes *katG* and *ahpC* are believed to be involved in the protection mechanisms used by the mycobacteria in defence against millimolar concentrations of hydrogen peroxide (Jackett, Aber, & Lowrie 1978; Manca et al. 1999). *M. tuberculosis* is naturally deficient in responding to oxidative stress as it lacks a functional *oxyR* (Deretic et al. 1995), which may also relate to the high sensitivity of *M. tuberculosis* to isoniazid (Dhandayuthapani et al. 1996). (section 1.11.2.2.d). *oxyR* is divergently transcribed from *ahpC* which encodes a close homolog of a alkylhydroperoxide reductase gene found in bacterial species involved in the reduction of substrate peroxides. It protects the cell by detoxifying hydrogen peroxide (Niimura, Poole, & Massey 1995), reactive nitrogen intermediates (Chen, Xie, & Nathan 1998) and reducing organic peroxides (Sherman et al. 1995). In *E. coli* the *oxyR* gene regulates the oxidative stress response. The *oxyR* gene product is a regulatory protein which is both a sensor of oxidative stress and a transcriptional activator of detoxifying enzymes including KatG and AhpC (Christman et al. 1985). The absence of *oxyR* in *M. tuberculosis* is thought to result in the silencing of *ahpC* in virulent strains of *M. tuberculosis* (Springer et al. 2001). *ahpC* is only feebly expressed but it remains functional. In isoniazid resistant *katG* deficient strains (Sherman et al. 1996) and sensitive strains which are exposed to isoniazid (Wilson et al. 1999) enhanced expression of *ahpC* is observed. Even though there is an absence of *oxyR* and a silencing of *ahpC* at different phases of growth *M. tuberculosis* can still protect itself due to overlap in *katG* and *ahpC* activity (Master et al. 2002). In Gram negative bacteria *oxyR* is a critical component of the oxidative stress response. *M. tuberculosis katG* and other parts of the oxidative stress response must be controlled

by an alternative factor to OxyR. Zahrt et al demonstrated that the catalase peroxidase protein is negatively regulated by FurA (Zahrt et al. 2001). The absence of a functional OxyR lends evidence to the argument that *M. tuberculosis* is relatively deficient in its defence against oxidative stress.

Another enzyme involved in the anti-oxidant defence system besides catalase is superoxide dismutase. These enzymes are metalloenzymes involved in the conversion of superoxide ( $O_2^-$ ) into hydrogen peroxide ( $H_2O_2$ ) and molecular oxygen ( $O_2$ ). *M. tuberculosis* *sodA* encodes a iron-cofactored superoxide dismutase and *sodC* encodes a copper and zinc superoxide dismutase (Andersen et al. 1991; Wu et al. 1998).

#### 1.14.2.3 Oxidative Damage to DNA

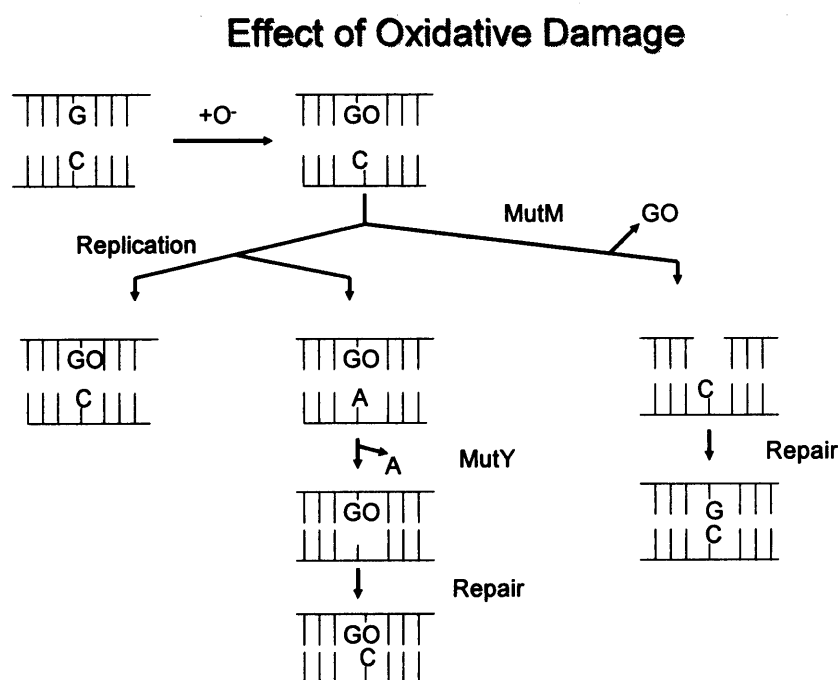
It has been shown both *in vitro* and *in vivo* that exposure to oxygen free radicals causes the oxidation of guanine residues in DNA generating 7,8-dihydro-8-oxodeoxyguanine (8-oxodG), generating what is termed a GO lesion. Normally guanine bases have a strong preference for cytosine during replication which is disrupted by 8-oxodG residues. DNA synthesis past the 8-oxodG can result in the misincorporation of adenine on the opposite strand. As a result the pairing of OG/A can arise in either strand during replication (Jackett, Aber, & Lowrie 1978). If this OG/A mismatch is not repaired, a further round of replication results in a  $G \rightarrow T$  mutation (Shibutani, Takeshita, & Grollman 1991). Adenine can be oxidised to form 8-oxodeoxyadenine (8-oxodA) which is less mutagenic than 8-oxodG and correctly pairs with thymine (Wood et al. 1992). However it has been shown to mispair with guanine *in vitro* (Shibutani et al. 1993).

#### 1.14.2.4 The GO Repair System

A high mutation rate has been demonstrated in a GO system knockout in *E. coli*. The GO repair system is composed of at least 3 proteins: MutM, MutY and MutT (Michaels et al. 1992). Knockout studies of the MutM and MutY proteins have shown their involvement in providing the organism with protection from the mutagenic effects of GO lesions (Boiteux & Laval 1983; Nghiem et al. 1988). Fowler et al showed a specific increase in A·T → C·G transversions in *mutT* strains, while showing an increase in G·C → T·A in *mutM* and *mutY* mutator strains (Fowler et al. 2003). The *mutT* product hydrolyzes 8-oxodGTP to prevent its use by DNA polymerase III holoenzyme (Fowler et al. 2003). An increase in G·C→T·A transversions has been characterised in a *mutY* mutator *E. coli* phenotype (Nghiem et al. 1988). *M. tuberculosis* is competent in repair of the effects of oxidative damage. MutM can remove oxidised guanine from the DNA and subsequent repair restores the original G/C pair (figure 1.9). If the GO lesion is not removed prior to replication, translesion synthesis can be accurate and result in C/GO which is a substrate for MutM. However more commonly translesion synthesis by replicative DNA polymerases is inaccurate, leading to the misincorporation of adenine opposite the GO lesion. The MutY protein removes this mispair and repair by polymerases results in a C/GO pair, which is a substrate for the MutM protein (Michaels et al. 1992). If the A/GO mismatch is not repaired, a further round of replication results in a G → T mutation. MutY can also participate in adenine excision from A:C mismatches (David & Williams 1998). The GO lesion may produce only a minor distortion of the DNA backbone so it is perhaps not the ideal substrate for nucleotide excision repair (Grollman & Moriya 1993).

Some strains of *M. tuberculosis*, notably the Beijing family, are associated with an increased risk of resistance and some consider this to be due to an increased mutation rate. Missense mutations in the three putative *mut* genes have been shown but no experimental evidence is available that directly links these lineages with an increased mutation rate (Rad et al. 2003).

**Figure 1.9:** Oxidative Damage can lead to GO lesions in DNA. These GO lesions can be removed by MutM, and repaired. If the lesion is not removed before replication, translesion synthesis can be frequently inaccurate leading to the misincorporation of A opposite the GO lesion, resulting in an A/GO pair which is a substrate for MutY. MutY removes the misincorporated adenine. Adapted from Michaels & Miller 1992.



#### 1.14.2.5 Other Mutagenic Effects on DNA

Besides the mutagenic effect of the 8-oxodG on the DNA, some studies have focused on the contribution of oxidised cytosines-5-hydroxycytosine (5-OH-C) and/or 5-hydroxyuracil (5-OH-U) to the high number of C→T transitions as a result of oxidative damage to DNA (Purmal, Kow, & Wallace 1994a; Purmal, Kow, & Wallace 1994b). It has been shown that oxidation of cytosine can give rise to 5,6-dihydroxy-5,6-dihydrocytosine (Cg), which can break further down to form 5-OH-C, 5-OH-U



and 5,6-dihydroxy-5,6-dihydrouracil (Ug) (Dizdaroglu et al. 1986). Kreutzer and Essigmann suggest a model for oxidative mutagenesis whereby the cytosine is oxidised followed by deamination to oxidised uracil species which is poorly repaired (Kreutzer & Essigmann 1998). This can result in strong miscoding during replication leading to a C → T or G → A transition unless the uracil is removed by a uracil DNA glycosylase encoded by the *ung* gene in *M. tuberculosis* (Handa, Acharya, & Varshney 2001). 5-OH-U and Ug are likely sources of the observed C → T transitions as they are present in oxidatively damaged DNA (Wagner, Hu, & Ames 1992). Both G·C and A·T base pairs are damaged by reactive oxygen species with about equal efficiency but recent studies suggest that G·C damage is more likely to lead to G·C → A·T transitions (Schaaper & Dunn 1991).

Methylation of guanine is a well known inducer of G·C → A·T transitions (Hoffmann, Crowley, & Theophiles 2002). In *E. coli* inadvertent methylations of this kind may occur when low levels of methyltransferase are present. However in *M. tuberculosis* 6-O methylguanine DNA methyltransferase, encoded by the *ada* gene, is expressed to repair alkylated guanine (Durbach et al. 2003).

### 1.14.3 Mechanism of Mutation Repair in *M. tuberculosis*

#### 1.14.3.1 The SOS Response

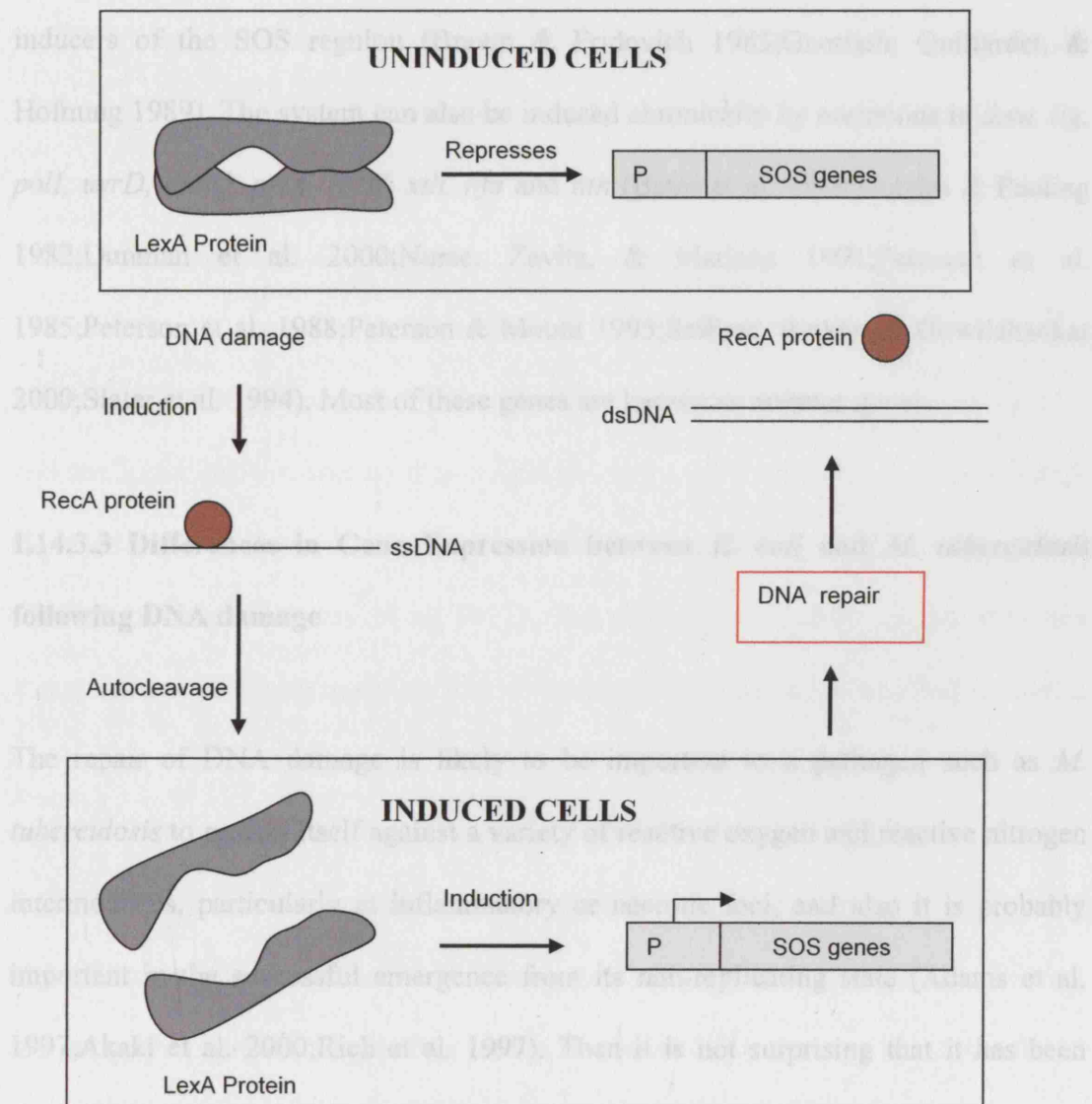
This system was originally discovered from studies that resulted from the exposure of *E. coli* to UV irradiation, where reactivation and mutagenesis increased of a UV irradiated phage λ when it infected a previously irradiated *E. coli* host (Weigle 1953).

Radman went on to propose that inducible SOS repair is related to mutagenesis as, upon induction, when the bacteria are exposed to stress, a number of genes that are normally in a repressed state are switched on to allow the repair of damaged DNA and reactivate DNA synthesis, and that ultimately these processes are linked to mutation (Radman 1974). He coined this phenomenon the SOS response, after Save Our Souls. It was then proposed that the SOS system is repressed by LexA protein and activated by RecA protein (Gudas & Pardee 1975) (figure 1.10). This system is the primary response for many bacteria to DNA damage (Friedberg, Walker, & Siede 1995). LexA is relatively stable in untreated cells where its main function is to act as a repressor of the SOS system. The target genes for LexA repression include many repair functions including *recA*, *recN*, *ruvAB*, *uvrAB*, *uvrD*, *dinB*, *umuDC* and DNA polymerase II (McKenzie et al. 2000). In total for *E. coli* there are 43 SOS genes inducible by DNA damage (Goodman 2002).

Under normal cellular conditions LexA binds to the SOS box, a specific sequence upstream of *lexA* and *recA* and all other genes belonging to the SOS system, and represses their transcription (Brent & Ptashne 1981; Little, Mount, & Yanisch-Perron 1981). The SOS genes are therefore normally repressed. When DNA is damaged, or when replication of DNA is blocked and single stranded DNA accumulates, RecA binds to these regions of DNA setting off a cascade of reactions. This binding of RecA occurs in the presence of a nucleoside triphosphate to form a nucleoprotein and converts RecA to an activated form, often referred to as RecA\* (Little et al. 1994). The levels of ATP and dATP during SOS induction are increased several fold (Barbe, Villaverde, & Guerrero 1983; Guerrero et al. 1984; Suzuki et al. 1983). This RecA\* serves as a co-protease to stimulate the autocatalytic cleavage of LexA (Little &

Mount 1982). These cleaved fragments of LexA can no longer bind to the SOS boxes (Bertrand-Burggraf et al. 1987). As the pool of intact LexA decreases, *recA* and other various SOS genes are expressed at an increased level (Walker 1984;Walker 1995). The affinity of a gene's SOS box for LexA, the location of the box relative to the promoter and the strength of this promoter, along with the presence of any constitutive promoters all influence the degree of induction of a gene (Friedberg, Walker, & Siede 1995;Schnarr et al. 1991).

**Figure 1.10:** The SOS response in *E. coli*. Under normal cellular conditions, LexA represses the SOS system. DNA damage serves as a distress signal, activates RecA which leads to the autocatalytic cleavage of LexA and induces a number of cellular functions involved in DNA repair. This repair can lead to mutagenesis. Once this damage is repaired, the SOS system is switched off and further mutagenesis ceases.



P – Represents the promoters of SOS genes

### 1.14.3.2 Induction of the SOS Response

Induction of the SOS system is caused by a variety of physiological states, changes in pH, starvation and stationary growth phase (Dri & Moreau 1994; Koch & Woodgate 1998; Taddei, Matic, & Radman 1995). The best documented physical agent to induce the SOS response is UV radiation. Mitomycin C and hydrogen peroxide are chemical inducers of the SOS regulon (Brawn & Fridovich 1985; Goerlich, Quillardet, & Hofnung 1989). The system can also be induced chronically by mutations in *dam*, *lig*, *poll*, *uvrD*, *dnaQ*, *priA*, *recN*, *xth*, *nfo* and *nth* (Bates et al. 1989; Condra & Pauling 1982; Dunman et al. 2000; Nurse, Zavitz, & Marians 1991; Peterson et al. 1985; Peterson et al. 1988; Peterson & Mount 1993; SaiSree, Reddy, & Gowrishankar 2000; Slater et al. 1994). Most of these genes are known as mutator genes.

### 1.14.3.3 Differences in Gene Expression between *E. coli* and *M. tuberculosis* following DNA damage

The repair of DNA damage is likely to be important to a pathogen such as *M. tuberculosis* to protect itself against a variety of reactive oxygen and reactive nitrogen intermediates, particularly at inflammatory or necrotic loci, and also it is probably important in the successful emergence from its non-replicating state (Adams et al. 1997; Akaki et al. 2000; Rich et al. 1997). Then it is not surprising that it has been shown to have homologs of genes required for the SOS response known to be involved in this response in *E. coli*, with the exception of *polB* (also known as *dinA*) the gene encoding DNA polymerase II, the UV mutagenesis gene *umuD*, although there are homologs of *umuC* (Mizrahi & Andersen 1998), *sulA*, the filamentation gene

and *himA*, the integration host factor gene (Brooks, Movahedzadeh, & Davis 2001). *dinX* (also known as *dinB2*) and *dinP* belong to the Y family of error-prone DNA polymerases and are probable homologs of *umuC*. Induction of these genes has not been observed upon exposure to hydrogen peroxide, mitomycin C and UV, making them apparently not part of the SOS regulon (Boshoff et al. 2003; Brooks, Movahedzadeh, & Davis 2001). Other significant differences exist; *ruvC* is induced by DNA damage in *M. tuberculosis* but not in *E. coli*. Also *recN* and *dinG* are induced in *E. coli* but not in *M. tuberculosis* (Brooks, Movahedzadeh, & Davis 2001).

#### 1.14.3.4 Binding of *lexA* and *recA*

It has been demonstrated also in *M. tuberculosis* that DNA damage can induce *recA* and the LexA protein can bind to a specific sequence upstream of *recA* and *lexA* (Movahedzadeh, Colston, & Davis 1997a; Movahedzadeh, Colston, & Davis 1997b; Papavinasasundaram et al. 1997). The consensus sequence of the SOS box varies from organism to organism and in mycobacteria it has been revealed to contain the pattern of TCGAACnnnnGTTCGA. In *M. tuberculosis* there are four potential upstream binding sites for LexA (Davis, Dullaghan, & Rand 2002; Dullaghan, Brooks, & Davis 2002). It may be possible that LexA binds to multiple sites, controlling some DNA damage inducible genes in *M. tuberculosis*, rather than to a single site allowing a more graded response depending on the degree of DNA damage. For some DNA damage inducible genes, *ssb* and *uvrA*, there are no possible SOS boxes upstream for LexA to bind (Brooks, Movahedzadeh, & Davis 2001). So there are DNA damage inducible genes that are regulated by *recA* which are entirely associated with SOS boxes and genes which are induced independently of RecA and LexA (Rand et al.

2003). These mechanisms have been demonstrated to occur in a *recA* mutant strain of *M. tuberculosis* (Davis et al. 2002). So perhaps there are multiple mechanisms for DNA damage induction in *M. tuberculosis*.

#### **1.14.3.5 Other Repair Systems**

##### **1.14.3.5.a Recombinational Repair**

The *M. tuberculosis* RecA is involved in recombinational repair as are RecBCD (exonuclease V). The RecBCD protein unwinds and degrades DNA; functioning as a helicase and has exo- and endo-nucleolytic activities for single stranded DNA, all requiring ATP (Kowalczykowski et al. 1994). Induction of nalidixic acid requires *recBCD* (McPartland, Green, & Echols 1980;Smith 1988). It is thought that one of the activities of RecBCD is the induction of the SOS response. *recB* and *recC* null mutants are sensitive to DNA damaging agents and after treatment with nalidixic acid are unable to derepress the SOS response (Gudas & Pardee 1976;Smith 1988). *recF* is involved in recombination and can be induced by the SOS system. RecF has the ability to bind to single stranded DNA and ATP (Salazar et al. 1996). However the absence of *recE* in *M. tuberculosis* may mean that this RecF pathway for repair is redundant (McFadden 1996).

##### **1.14.3.5.b Base Excision Repair**

Base excision repair involves the cleavage of the glycosidic bond connecting a damaged base to the DNA sugar phosphate backbone to repair the damage caused by exposure to reactive oxygen intermediates and other reactive metabolites (Lindahl 1993;Sancar 1994). It is a two step pathway; recognition and removal of a specific

modified base(s) by specific DNA glycosylases leaving apurinic/aprimidinic (AP) sites in the DNA, followed by cleavage of the DNA, excision of the resulting sugar phosphate residue, repair mediated by DNA polymerase and subsequent ligation of the phosphodiester backbone (Seeberg, Eide, & Bjoras 1995). There are a number of genes involved in this repair pathway: *nei* and *nth* involved in the repair of oxidised pyrimidines, *fpg* which repair purines and the AP-endonuclease-encoding genes *xthA* and *nfo*. Also the GO system, involving *mutY*, *mutM* and *mutT* is responsible for the protection against the mutagenic effects caused by GO lesions, created from 8-oxodG residues (section 1.14.2.4).

#### 1.14.3.5.c Nucleotide Excision Repair

Genes involved in nucleotide excision repair are present in the *M. tuberculosis* genome which are ultimately responsible for excising the section of DNA containing the damaged site and replacing it with normal DNA (Sancar 1994). This repair system involves an excinulease that can detect thymine dimers composed of the *uvrA*, *uvrB* and *uvrC* subunits. The genes *uvrD1* and *uvrD2*, both encoding DNA helicase II are also involved in nucleotide excision repair. In addition to DNA polymerase activity, the DNA polymerase I encoded by *polA*, has exonuclease activity both in the 3' - 5' (Huberts & Mizrahi 1995) and possibly in the 5' - 3' direction. DNA polymerase I is an important contributor in DNA repair functions and in genome stability, especially during stationary phase of the bacterial life cycle (Arrigo, Singh, & Modak 2002).



#### 1.14.3.5.d Mismatch Repair

Mismatches occur as a result of non-homologous recombination, replication error or damage to DNA bases and the mismatch repair system corrects these errors. The genome sequence of *M. tuberculosis* has revealed that it is devoid of genes encoding the mismatch repair proteins MutS, MutH and MutL (Cole et al. 1998; Mizrahi & Andersen 1998). Springer et al provide biological evidence of the lack of mismatch repair in *M. smegmatis* (Springer et al. 2004). The absence of this repair system may explain the slow processivity in the mycobacterial DNA polymerase, which is 20 times slower than in *E. coli* (Hiriyanna & Ramakrishnan 1986) and why slowing the rate of DNA synthesis may increase the fidelity of DNA polymerases (Radman 1998). *M. tuberculosis* also lacks *dcm* and *vcr* genes. These function in short-patch mismatch repair in *E. coli*.

#### 1.14.4 Mutation Rates

##### 1.14.4.1 Determining Mutation Rates

Measurement of mutation rates has become of great importance in medical microbiology by providing a useful tool when comparing the likelihood of the development of anti-microbial resistance among bacterial strains. Mutations that arise early in a broth culture will produce a large clone of identical descendants, a “Jackpot culture” (Luria & Delbrück 1943). Thus the size of the mutant progeny depends on when the mutation occurs. Early mutations are rare, the final numbers of mutants in a series of parallel cultures can be compared and would have “a distribution with an

abnormally high variance” (Luria & Delbrück 1943). *In vitro* mutation rates can be investigated by measuring the frequency that detectable mutations arise in a bacterial population in the presence of a given antibiotic concentration, which may be twice the known MIC for that bacterium. Mutation frequency has been more often determined than the mutation rate. It is the average of a fraction of bacterial cultures that have mutated in few replicate cultures. Mutation rate can be defined as the probability of a cell in sustaining a mutation during exponential growth. This probability may be low but it is not zero. When the mutation rate is properly determined then it is more accurate and reproducible than the mutation frequency and it also gives more information about biological processes (Rosche & Foster 2000). When measuring the mutation frequency, low probability events occurring at early stages of the growth of the population have huge consequences. This makes the measurement of the mutation frequency inaccurate as it does not reflect the stochastic process of mutant accumulation.

Fluctuation analysis allows the measuring of mutation rates *in vitro* by determining the distribution in parallel cultures of mutant numbers and analysing that distribution. A series of pivotal experiments by Luria and Delbrück in 1943 pioneered this method (Luria & Delbrück 1943). These experiments aimed at validating the hypothesis that the transformation of bacteria into phage resistant bacteria arises from spontaneity (random mutation) and not from adaptation (directed mutation). They set about to determine the rate at which the transformation takes place. In this experiment, sensitive *E. coli* cells were cultured. Samples were divided and tested for the mutant phenotype by growth on agar plates containing bacteriophage T1 which is ultimately lethal for the bacteria. It was found that resistant colonies were present in the

inoculum before exposure to the phage. Thus the bacteria did not need to be challenged by the phage before becoming resistant but developed this resistance spontaneously. Luria and Delbrück suggested two methods for estimating the overall mutation rate for the population: (1) the  $P_0$  method which is based on the proportion of cultures with no mutants observed and (2) the method of the mean, which relies on the determination of the mean number of mutants (table 1.4). Both of these methods assume a Poisson distribution with a mean and variance equal to the product of the probability of mutation of a bacterium and the number of bacteria that is the ratio of variance to mean is one. The first method to calculate the Luria and Delbrück distribution was provided by Lea and Coulson who used the median number of mutants to calculate mutation rates which is far more accurate than the method of the mean (Lea & Coulson 1949) (table 1.4). Other methods have used the average number (Capizzi & Jameson 1973). A hypothesis suggested in 1988 deviates from the formulas of Lea and Coulson and suggests that besides mutations occurring at random, other mutations can be driven by the 'needs' of the bacteria themselves (Cairns, Overbaugh, & Miller 1988).

**Table 1.4:** Parameters for the methods used in determining mutation rates (Rosche & Foster 2000).

Method	Range	Constraints	Ref
The $p_0$ method	$0.1 \leq p_0 \leq 0.7$ $0.3 \leq m \leq 2.3$	Highly variable results	(Luria & Delbrück 1943)
Luria and Delbrück method of the mean	None	Uneven distribution of mutant numbers	(Luria & Delbrück 1943)
Lea-Coulson method of the median	$1.5 \leq m \leq 15$	More accurate than Luria and Delbrück method	(Lea & Coulson 1949)
Drake formula	$30 \leq m$	Reports mutation frequencies	(Drake 1970)
Jones mediator method	Unknown but works when $1.5 \leq m \leq 10$	Unknown	(Jones, Thomas, & Rogers 1994)
The Quartiles method	$2 \leq m \leq 14$	Unknown	(Armitage 1952; Koch 1982)
MSS maximum likelihood method	All	Unknown	(Sarkar, Ma, & Sandri 1992)

#### 1.14.4.2 Design of Mutation Rate Experiments

There are three main parameters which must be considered for any mutation rate experiment (Luria & Delbrück 1943; Rosche & Foster 2000). Firstly  $m$ , the expected number of mutational events per culture should not be below 0.3. It is important to note that this parameter is not the number of mutants (cells carrying a given mutation) per culture but it is the number of mutation events.  $m$  will depend on the mutation rate and on the amount of cell growth. It is possible to vary  $m$  by plating different volumes of culture although this can introduce errors. The value for  $m$  will vary depending on the method used to calculate mutation rate and on the number of mutants per plate which are easily countable. For the Poisson distribution method,  $m$  should be greater than 0.3 but less than 2.3. For the method of the mean  $m$  should be between 1.5 and 15.

The second parameter for consideration is the number of parallel cultures,  $C$ , to be set up to represent the bacterial population sufficiently. It is recommended that a precision level of 20% is acceptable for the estimate of  $m$ . For example the  $p_0$  method ( $0.3 \leq m \leq 2.3$ ), to get a  $m$  value of 1.5 then 50 parallel cultures must be used (Rosche & Foster 2000) (table 1.4). The precision level in this case is a measure of the reproducibility of results rather than a measurement of accuracy (measurement of how well the estimated  $m$  and actual  $m$  compare).

A final important parameter to consider is the initial inoculum,  $N_0$ , to use in the experiment. It is important that this number is enough to represent the population but not too high so as to generate too many mutants and also that it does not contain any

pre-existing mutants. At the other end of the scale a small inoculum will mean a longer incubation period, which is far from ideal when working with slow growing *M. tuberculosis* cultures. It is important that final number of cells in a culture,  $N_t$ , is the same in each replicate experiment. In mutation rate experiments cultures are grown to saturation before mutant selection but in some experiments exponentially growing cells must be used. In all cases the value of  $N_0$  must be negligible relative to  $N_t$ . Before mutant selection, plate counts can be done to measure the total number of cells in the cell deposit. This will ensure that numbers are not variable between replicate experiments. Variations in  $N_t$  can be eliminated by using a large  $N_0$  that is possible without introducing a mutant. Rosche and Foster suggest that a good rule of thumb is to use an initial inoculum of about  $mN_t/10^4$ . Every effort must be put in to ensure that all these parameters remain constant from experiment to experiment.

Methods for determining the mutation rate,  $\mu$ , are outlined in table 1.4. Each method can be used for different values of  $m$  and the most vital part of determining a mutation rate for a bacterial culture is the selection of which method to use. Some preliminary experiments to determine the range of the number of mutants obtained when a given number of cells are plated can be performed to establish the method of choice.

The  $p_0$  method should only be used when  $0.1 \leq p_0 \leq 0.7$ , the number of cultures with no mutants is counted and the following equation is used to calculate  $p_0$ .

$$p_0 = \frac{\text{no. of cultures with no mutants}}{\text{total no. of broth cultures}}$$

The number of mutations per culture ( $m$ ) is then calculated from the following formula:

$$m = -\ln(p_0)$$

The  $p_0$  method is best used for the range of  $0.3 \leq m \leq 2.3$ . The mutation rate ( $\mu$ ) is then calculated:

$$\mu = \frac{m}{\text{average cfu/mL}}$$

where average cfu/mL (colony forming units/mL) is usually calculated from plate counts performed on the original broth culture.

#### 1.14.4.3 Assumptions of Fluctuation Analysis

There are a number of assumptions that Luria and Delbrück's analysis makes. It is assumed that all bacteria have grown from a single genotype, divisions are synchronous and do not vary during the growth of the culture, no cell death and reversion of mutants occurs, mutation only occurs during cell division and results in only one mutant (proportion of mutants is always small), growth rates of mutants and non-mutants are the same, the initial number of cells is negligible relative to the final number of cells, all mutants are detected and no mutants arise after selection is imposed. Bacterial growth is never synchronized and they will be at different points in their division cycles at any given time. Haldane set about to improve Luria and Delbrück's model by considering plating a sub-population and calculating the moments of the distribution (Sarkar 1991). He also considered nonsynchronous divisions, the effect of cell death and suggested that mutants have a lower mutation frequency than susceptible cells which may happen because of a fitness deficit or a

high reversion rate which is less likely. A factor ignored by Haldane was considered by Armitage in 1952 which is phenotypic lag, a concept that late mutants may fail to produce viable colonies (Armitage 1952).



### 1.15 Fitness

The distribution of mutational effects on fitness and the mutation rate can determine the fate of populations of bacteria (Lynch, Conery, & Burger 1995). Resistance to antibiotics is assumed to be usually associated with a fitness cost. This initial cost can be rapidly reduced by compensatory mutations which are important for adaptation and evolution of most organisms. In pathogenic organisms fitness is a measure of the organism's ability to survive, reproduce and be transmitted. Resistance to antibiotics, in *M. tuberculosis* occurs by genomic mutations, can result in the impairing of vital functions or can confer metabolic burdens (Maisnier-Patin & Andersson 2004). When the selective pressure exerted by the drug is removed, the resistant organisms with reduced fitness may become extinct or to ensure survival, may revert back to wild type or compensatory mutations may occur. Resistant strains may have slower growth rates than sensitive strains. It has been shown that compensation is more likely than reversion in reducing the deleterious cost of mutation (Andersson & Levin 1999; Bleiber et al. 2001; Maisnier-Patin et al. 2002; Moore, Rozen, & Lenski 2000; Reynolds 2000).

The idea that resistance is not associated with a reduced bacterial fitness has been studied. Some resistance mutations appear to confer no cost as measured *in vitro*. Pym et al showed that the common Ser315Thr mutation in *katG* of *M. tuberculosis* can confer isoniazid resistance with minimal reduction in fitness if any (Pym, Saint-Joanis, & Cole 2002). This hypothesis can be supported by the fact that it is the most frequently isolated mutation in clinical isolates of *M. tuberculosis* (Ramaswamy & Musser 1998). In 1953 studies by Middlebrook reported that isoniazid resistant strains

in guinea pigs were less pathogenic or had a slower growth rate than parent isoniazid sensitive strains (Middlebrook & Cohn 1953). Work by Ordway et al in a study of 15 clinical isolates of *M. tuberculosis* found that drug resistant strains had a range of virulence for mice (Ordway et al. 1995). These findings support the hypothesis that isoniazid resistant strains are heterogeneous in terms of resistance and pathogenicity, which is also confirmed by Middlebrook. They did not find that increased drug resistance is associated with loss of virulence of the isolate. However Middlebrook's findings are also evidence that the acquisition of isoniazid resistance attenuates *M. tuberculosis*. Another study correlated the level of isoniazid resistance to the virulence of the organism, with highly resistant strains showing less virulence (Barnett, Busby, & Mitchison 1953). Mitchison et al reported a significant proportion of clinical strains from patients in India which were isoniazid resistance also were of low virulence in a guinea pig model (Mitchison et al. 1960). Certain *rpsL* mutations in streptomycin resistant isolates of *M. tuberculosis* (Bottger et al. 1998), *E. coli* (Schrag & Perrot 1996; Schrag, Perrot, & Levin 1997) and *Salmonella typhimurium* (Bjorkman et al. 2000; Bjorkman, Hughes, & Andersson 1998) appear to confer no fitness cost.

Billington et al used an *in vitro* competitive assay to determine the relationship between *rpoB* mutations conferring resistance to rifampicin in *M. tuberculosis* and the relative fitness of these mutations (Billington, McHugh, & Gillespie 1999). They found that rifampicin resistance was associated with a reduction in fitness but for certain substitutions (the Ser531Leu) the fitness cost conferred in comparison to the parent wild type was relatively low. This substitution is the most frequent *rpoB* mutation in rifampicin resistant clinical isolates of *M. tuberculosis* (Ramaswamy & Musser 1998). They correlated relative fitness with the frequency that the mutation

was isolated in a clinical setting. The data from this paper suggest that mutants isolated more frequently in clinical practice have a higher relative fitness on initial isolation. Perhaps there are compensatory mutations which are occurring and therefore there is a lower likelihood of spontaneous reversion to susceptibility.

Other work has shown that a multidrug isolate of *M. tuberculosis* had decreased fitness when compared to a sensitive organism which suggests that the fitness deficit implies a physiological cost for the development of drug resistance (Davies et al. 2000a). This multidrug resistant isolate did not lose its capability to cause disease and remained virulent. It is possible to conclude that the measure of fitness in this assay may have little bearing on the virulence of the organism *in vivo*, in its natural host. Clearly, studies investigating fitness in correlation with resistance are very variable in their findings depending on the assay conditions used. In *S. aureus* the biological cost of rifampicin resistance has been studied (Wichelhaus et al. 2002). It has been shown that mutations in *rpoB* were associated with a considerable loss in fitness *in vitro*, which may differ *in vivo*. *In vivo* it appears that the variation of mutations in *rpoB* in clinical isolate of *S. aureus* depends on the relative Darwinian fitness of the organism.

### 1.16 Aims of thesis

To investigate the increase in the emergence of antibiotic resistance in tuberculosis by analysing what drives the increase in mutation is the overall purpose of this thesis. Whether it is due to intrinsic factors such as faulty DNA repair mechanisms (investigated in chapter 6, looking at the SOS response increasing mutation upon sub-MIC quinolone exposure) or other factors such as the exposure of the bacilli to oxidative stress making them more susceptible to mutation (investigated in chapter 5) remains to be elucidated. Resistance acquisition *in vivo* and *in vitro* leads to a reduction in relative fitness so sensitive strains should predominate and outgrow the resistant strains. Mutation acquisition in clinical isolates is examined in chapter 4. However the resistant strains have the ability to adapt to eliminate their fitness deficit through the acquisition of compensatory mutations through continuous mutation thereby leading to the presence of mixed populations of sensitive and resistant bacteria, investigated in chapter 3.

## **Chapter 2: General Materials and Methods**

### **2.1 Preparation of culture**

#### **2.1.1 Middlebrook 7H9 broth**

4.7g Middlebrook 7H9 powder (BD, Le Pont de Claix, France) was dissolved in 900mL distilled water in a litre bottle. 2mL Tween 80 [polyoxyethylene (20) sorbitan mono-oleat] (BDH, VWR International Ltd., Poole, England) was added and the bottle was placed in a 55°C water bath for 20 min to dissolve the powder and tween. This was aliquoted into 5 x 180mL and autoclaved at 121°C, 15psi for 15 min. The medium was stored at room temperature. Before use, 20mL Middlebrook albumin dextrose catalase (ADC) enrichment (BD, Le Pont de Claix, France) was added to each aliquot.

##### **2.1.1a Albumin Dextrose Enrichment**

8.1g sodium chloride (BDH, VWR International Ltd., Poole, England), 50g bovine albumin fraction V (Sigma Aldrich, Steinheim, Germany) and 20g D-glucose (BDH, VWR International Ltd., Poole, England) were dissolved in 1L of distilled water. This was filter sterilised through 0.2µm pores (Nalgene, supplied by Fisher Scientific, Leicestershire, UK) and stored at 4°C. An aliquot equivalent to 10% of total volume of this enrichment was added to 7H9 broth.

### **2.1.2 Middlebrook 7H10 agar**

3.8g Middlebrook 7H10 powder (BD, Le Pont de Claix, France) was dissolved in 180mL distilled water. 0.5mL glycerol (Sigma-Aldrich, Steinheim, Germany) was added and autoclaved at 121°C, 15psi for 15 min. The agar was cooled to 50°C and 20mL Middlebrook oleic albumin dextrose catalase (OADC) enrichment (BD, Le Pont de Claix, France) was added. The bench top was wiped with 4% hycolin solution (Adams Healthcare, Leeds, UK). 20mL liquid media was poured into sterile disposable petri dishes (Sterlin, supplied by Western Laboratory Services) in between 2 bunsen flames. The plates were left to dry on a flat surface and stored face down in plastic bags at 4°C for no longer than 3 days. Plates were dried, lids off, face down, at 37°C for 20 min or until all the condensation had evaporated before use.

### **2.1.3 Mueller Hinton broth**

22g Mueller Hinton powder (BD, Le Pont de Claix, France) was dissolved in 1L distilled water. 4mL of Tween 20 [polyoxyethylene (20) sorbitan monolaurate] (BDH, VWR International Ltd., Poole, England) was added and autoclaved. The broth was stored at room temperature.

### **2.1.4 Mueller Hinton agar**

38g Mueller Hinton agar powder (BD, Le Pont de Claix, France) was dissolved in 1L distilled water and autoclaved. The media were equilibrated to 50°C in a water bath. The bench top was wiped with 4% hycolin solution (Adams Healthcare, Leeds, UK).

20mL liquid media was poured into sterile disposable petri dishes (Sterlin, supplied by Western Laboratory Services) between 2 bunsen flames. Plates were cooled on a flat surface and stored face down in plastic bags at 4°C. Plates were dried before use at 37°C for 30 min or until no condensation was seen.

### **2.1.5 Blood Agar**

Ready prepared Columbia agar with horse blood (Oxoid Ltd., Hampshire, UK) were used.

## **2.2 Preparation of buffers and solutions**

### **2.2.1 0.5M EDTA**

186.1g EDTA disodium salt was dissolved in 800mL distilled water and adjusted to pH 8.0 with NaOH (approx 20g NaOH pellets) (Sigma Aldrich, Steinheim, Germany) and stirred vigorously.

### **2.2.2 1M Tris**

121.1g Tris base (Promega, Hampshire, UK), 42mL concentrated HCl stock was dissolved in 1L distilled water and adjusted to pH 8.0.

### **2.2.3 5M NaCl**

146.1g sodium chloride (BDH, VWR International Ltd., Poole, England) was dissolved in 500mL distilled water.

#### **2.2.4 TE buffer**

10mL 1M Tris pH 8.0 and 2mL 0.5M EDTA was made up to 1L with distilled water.

#### **2.2.5 10% SDS**

10g SDS (Sigma Aldrich, Steinheim, Germany) was dissolved in 100mL distilled water.

#### **2.2.6 CTAB/NaCl**

4.1g NaCl (BDH, VWR International Ltd., Poole, England) and 10g CTAB (Cetyltrimethylammonium bromide) (Sigma Aldrich, Steinheim, Germany) were dissolved in 100mL distilled water. The reagent was heated to 65°C to dissolve, if necessary.

#### **2.2.7 Lysozyme**

100mg lysozyme (Sigma Aldrich, Steinheim, Germany) was dissolved in 10mL sterile distilled water to prepare a 10µg/mL solution. 1mL aliquots were stored at -20°C.

#### **2.2.8 Proteinase K**

10mg/mL stock solution was prepared by dissolving 100mg proteinase K (Promega, Hampshire, UK) in 10mL sterile distilled water. 1mL aliquots were stored at -20°C .

#### **2.2.9 SDS/Proteinase K**

5µL proteinase K (10mg/mL) was added to 70µL 10% SDS.



**2.2.10 Tris-Borate EDTA buffer (TBE)**

5 x solution was prepared by mixing 54g Tris base (Promega, Hampshire, UK), 27.5g boric acid (BDH, Leicestershire, UK) and 20mL 0.5M EDTA pH 8.0 in 1L distilled water. A magnetic heated stirrer and flea were used until the buffer had dissolved.

**2.2.11 5M Guanidine thiocyanate (GTC) solution**

295.4g guanidine thiocyanate (Promega, Wisconsin, USA) was dissolved in 200mL distilled water. The solution was shaken well and left in a hot room overnight. If the GTC was not dissolved overnight, the solution was shaken again and left in hot room for another 30 min. 2.5g sodium N-lauroyl sarcosine (Sigma Aldrich, Steinheim, Germany), 12.5mL 1M Sodium citrate pH 7.0 and 5mL tween 80 (BDH, VWR International Ltd., Poole, England) were added to the solution. The solution was made up to a final volume of 500mL with distilled water. 3.5mL  $\beta$ -mercaptoethanol (Sigma Aldrich, Steinheim, Germany) was added immediately prior to use in a fume hood. The solution minus the  $\beta$ -mercaptoethanol was stored at room temperature for ~ 3 months.

### 2.3 Miles and Misra Plate Count

This technique was devised by Miles & Misra (1938) to perform a viable bacterial count by calculating the number of colony forming units from a small drop of broth diluent. This is done by counting the number of colonies which grow on an agar plate from the drop and calculating per mL.

*M. tuberculosis* was cultured in Middlebrook 7H9 broth containing Tween 80 to prevent clumping (section 2.1.1). Using a 1mL fine needle syringe the broth culture was passed through 6 times to get a single cell suspension prior to dilution. The broth suspension was serially diluted in Middlebrook 7H9 broth to form a dilution series down to  $10^{-6}$ . The dilutions were vortexed briefly and 20 $\mu$ L or 50 $\mu$ L spots were plated from a height of 2.5cm on Middlebrook 7H10 agar (section 2.1.2). The plates were incubated for 2 weeks at 37°C.

After 2 weeks, colonies were counted for each dilution which had been plated. Dilutions where more than 20 colonies are present without any confluence were used to make the viable count. Colony forming units per mL were calculated by the following equation:

$$x = \frac{n y}{z}$$

x = colony forming units per mL

n = mean number of colonies per 3 counts

y = dilution factor

z = dilution at which the colonies were counted

## 2.4. Minimum inhibitory concentration (MIC) determination

### 2.4.1 Determination of the MIC of moxifloxacin for *M. fortuitum* NCTC 10394

1. *M. fortuitum* NCTC 10394 was obtained from the National Collection of Type Cultures (NCTC, Colindale, London, UK) and was stored on blood agar plates.
2. A loopful of bacteria from a plate was inoculated into 10mL Mueller Hinton (MH) broth (section 2.1.3) and incubated at 37°C.
3. Moxifloxacin (gift from Bayer, Dusseldorf, Germany) was prepared in sterile distilled water to a stock concentration of 1mg/mL and stored for several weeks in 1mL aliquots at -20°C.
4. Mueller Hinton agar plates containing moxifloxacin at 0.015, 0.03, 0.06, 0.12, 0.25, 0.5µg/mL were prepared (section 2.1.4). Control plates without drug were also prepared.
5. Broth was centrifuged at 3,000 g for 10 min.
6. The supernatant was discarded and the pellet was resuspended in 1mL MH broth by a brief vortex.
7. The resuspended pellet was diluted  $10^{-1}$  to  $10^{-5}$  in MH broth.
8. Twenty microlitres of dilutions  $10^{-3}$  to  $10^{-5}$  were dropped onto drug containing- and drug free- plates.
9. The spots were left to absorb and then incubated for 48hr at 37°C.
10. The MIC value was recorded as the lowest concentration of antibiotic that exhibited no bacterial growth.

#### 2.4.2 Determination of MIC's using the multi-inoculator system

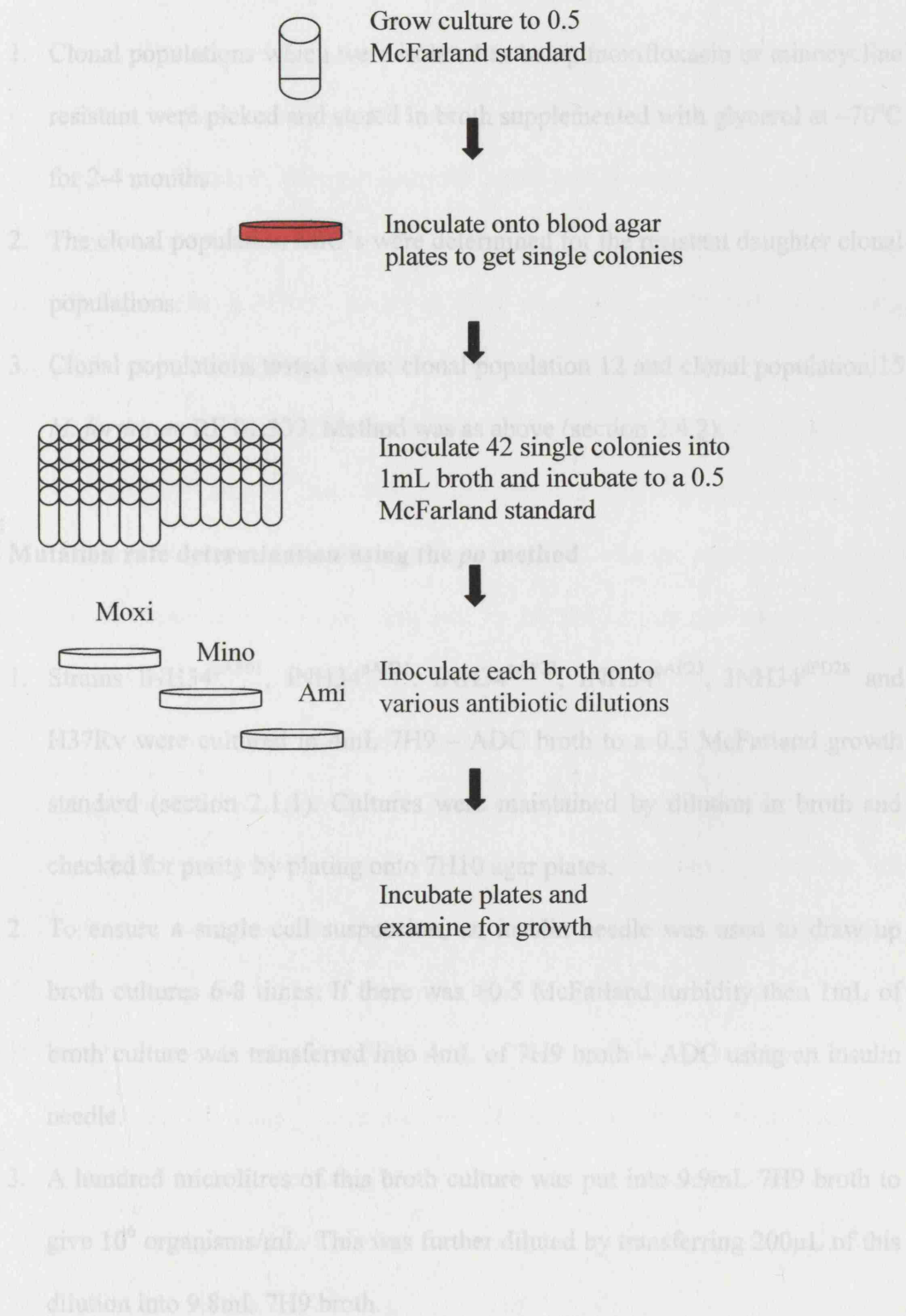
1. Bacteria: *M. fortuitum* NCTC 10394, *M. fortuitum* clinical isolates RF 01:332, RF 02:159 and RF 02:173, *M. smegmatis* NCTC 8159 and *M. smegmatis* mc<sup>2</sup>155 were used in these experiments. All clinical isolates were cultured from specimens submitted to the Department of Medical Microbiology, Royal Free Hospital, London, UK. They were multiple isolates from the same patient. *M. fortuitum* RF 01:332 was isolated prior to drug treatment and it is multi-drug resistant. The isolates 02 were from the following year. *M. smegmatis* mc<sup>2</sup>155 was obtained from the Institute of Pasteur, Paris.
2. Antibiotic preparations used were obtained as powder substances; moxifloxacin (Bayer), amikacin and minocycline (Sigma, Poole, UK). Sterile water was added to each antibiotic to give a final concentration of 1mg/mL. This sterile stock solution was divided into 1ml aliquots and stored at -20°C for several weeks.
3. All strains and clinical isolates were maintained for short term storage on blood agar plates at 4°C (section 2.1.5) and for longer term storage in MH broth supplemented with glycerol at -70°C. Both storage methods were used in this experiment.
4. Bacteria were inoculated into MH broth containing 0.25% Tween 20 to prevent clumping, from blood agar plates and grown to a turbidity of > 0.5 McFarland ( $1.5 \times 10^8$  organisms/mL) (figure 2.1). The growth in the broth was compared visually to the 0.5 McFarland standard.

5. Broth was inoculated onto horse blood agar plates in order to get single colonies (if there was difficulty in getting single colonies this was achieved by plating a 1/10 or 1/100 or 1/1000 dilution of the broth culture).
6. Plates were incubated for 2-3 days at 37°C.
7. Forty two single colonies were picked and inoculated into 1mL MH broth in 1.5mL microcentrifuge tubes and incubated until > 0.5 McFarland growth was observed. This took 2-3 days.
8. Each broth culture was inoculated on plates containing moxifloxacin or minocycline 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2µg/mL, or amikacin 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7µg/mL and on control drug free plates, using a multi-inoculator system (Denley). This system inoculates approximately 3µL of each broth culture, containing approximately  $5 \times 10^5$  organisms/mL, onto the agar plate.
9. The plates were incubated for 48 hrs and examined for growth or no growth.

**Table 2.1:** Experimental replicates for each strain and drug.

Isolate	No. of replicates		
	Moxifloxacin	Minocycline	Amikacin
<i>M. fortuitum</i> NCTC 10394	1	0	0
<i>M. fortuitum</i> RF 01:332	3	3	4
<i>M. fortuitum</i> RF 02:159	3	1	1
<i>M. fortuitum</i> RF 02:173	1	1	1
<i>M. smegmatis</i> NCTC 8159	2	2	2
<i>M. smegmatis</i> mc <sup>2</sup> 155	1	1	1

**Figure 2.1:** Schematic representation of the method for determining clonal population MIC's within a broth culture of an isolate.



### 2.4.3 Isolation of resistant daughter clonal populations and subsequent MIC determination

1. Clonal populations which were isolated as being moxifloxacin or minocycline resistant were picked and stored in broth supplemented with glycerol at  $-70^{\circ}\text{C}$  for 2-4 months.
2. The clonal population MIC's were determined for the resistant daughter clonal populations.
3. Clonal populations tested were: clonal population 12 and clonal population 15 *M. fortuitum* RF 01:332. Method was as above (section 2.4.2).

### 2.5 Mutation rate determination using the *po* method

1. Strains INH34<sup>pAP01</sup>, INH34<sup>pAP21</sup>, INH34<sup>pAP22</sup>, INH34<sup>pAP23</sup>, INH34<sup>pPD28</sup> and H37Rv were cultured in 4mL 7H9 – ADC broth to a 0.5 McFarland growth standard (section 2.1.1). Cultures were maintained by dilution in broth and checked for purity by plating onto 7H10 agar plates.
2. To ensure a single cell suspension, an insulin needle was used to draw up broth cultures 6-8 times. If there was  $>0.5$  McFarland turbidity then 1mL of broth culture was transferred into 4mL of 7H9 broth – ADC using an insulin needle.
3. A hundred microlitres of this broth culture was put into 9.9mL 7H9 broth to give  $10^6$  organisms/mL. This was further diluted by transferring 200 $\mu\text{L}$  of this dilution into 9.8mL 7H9 broth.

4. This was then distributed into 28 microcentrifuge tubes with 750 $\mu$ L 7H9 broth – ADC. Two hundred and fifty microlitres was added to each tube to give approx 5,000 cells/mL.
5. These cultures were incubated for 2 weeks at 37°C with daily gentle agitation.
6. 7H10 agar plates were prepared containing 10% OADC (section 2.1.2) and 2 $\mu$ g/mL rifampicin (Sigma Aldrich). Also plates without the drug were prepared.
7. After 2 weeks, a Miles and Misra plate count was performed on 3 broths (Miles & Misra 1938) (section 2.3). Dilutions  $10^{-1}$  up to  $10^{-6}$  were prepared, 3 plates for each set of plate counts were used. Twenty microlitres of dilutions  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  were spotted in triplicate onto 7H10 agar plates.
8. Before plating, the plates were incubated at 37°C with the agar face down and the lid removed for 30 min. This was to dry the surface and ensure that the drops inoculated will soak into the agar.
9. The remaining 25 broth cultures were centrifuged at 12,200 g for 5 min and the supernatant was poured off. They were then gently spun for 1 min to ensure all the liquid was at the bottom of the tube. Then the total volume left in the tube was plated on 2 $\mu$ g/mL rifampicin 7H10 agar plates (approx. 40 $\mu$ L). Each plate was divided into 4 sections, with one plate divided into 5.
10. All plates were incubated at 37°C upright overnight and inverted the next day. Growth for the plate counts was usually observed after 2 weeks, whereas growth on the drug containing plates usually took 5-6 weeks incubation.
11. Results were gathered and colony forming units per mL (x) were calculated as described in section 2.3.



12. The proportion of cultures with no mutants were calculated ( $p_0$ ) i.e. that had no growth on rifampicin (section 1.14.4.2).

$$p_0 = \frac{\text{no. of cultures with no mutants}}{\text{total no. of parallel cultures}}$$

13. The number of mutations per culture ( $m$ ) was calculated from the following formula:

$$m = -\ln(p_0)$$

14. The mutation rate ( $\mu$ ) was calculated:

$$\mu = \frac{m}{x}$$

15. These experiments were repeated 3 times for each strain.

### 2.5.1 Mutation rate determination following exposure to hydrogen peroxide

The strains INH34<sup>pAP01</sup>, INH34<sup>pAP23</sup> and H37Rv were exposed to oxidative stress. The experiment 2.5 was followed exactly as above up to step 6 but included additional steps.

- a) After 2 weeks incubation, the 28 broths were centrifuged and the supernatant poured off. The pellets were re-suspended in 500 $\mu$ L of 8mM H<sub>2</sub>O<sub>2</sub> 7H9 – ADC broth.
- b) The tubes were gently vortexed and re-incubated for 24 hours.
- c) Steps 6-15 of method 2.5 were followed.

## 2.6 Determination of mutation frequency after exposure to oxidative stress

1. Strains *M. tuberculosis* INH34<sup>pAP01</sup>, INH34<sup>pAP23</sup> and INH34<sup>pPD28</sup> were cultured in 10mL 7H9 broth – ADC broth to a 0.5 McFarland growth standard (approximately 2 weeks later) and checked for purity by plating onto 7H10 agar.
2. A Miles and Misra plate count was performed on each culture (section 2.3).
3. Each culture was spilt into 5mL aliquots and centrifuged at 3,000 g for 30 min.
4. The supernatant was discarded and the pellet was re-suspended in 5mL 7H9 broth.
5. The tubes were vortexed briefly and centrifuged at 3,000 g for 30 min.
6. Step 3 – 4 were repeated to wash away the catalase which was in the original broth culture.
7. The supernatant was discarded and the pellets were re-suspended in 5mL 7H9 broth or 5mL 7H9 broth containing 8mM H<sub>2</sub>O<sub>2</sub>. The cultures were incubated for 24hr at 37°C.
8. The cultures were centrifuged at 3,000 g for 30 min.
9. The supernatant was discarded and each pellet was washed with 1mL 7H9 broth.
10. Each pellet was re-suspended in 0.5mL 7H9 broth and plated onto 7H10 agar plates containing 2µg/mL of rifampicin. A Miles and Misra plate count was performed on 0.1mL.
11. This experiment was repeated 3 times for each strain.

## 2.7 Nucleic Acid Extraction and Clean-up

### 2.7.1 Isolation of genomic DNA

1. From a well grown LJ slope culture of *M. tuberculosis* or a well grown MH plate of *M. fortuitum* at least 1 loopful of organisms were harvested and transferred to a sterile 2mL polypropylene microcentrifuge tube (Sarstedt Ltd., Leicester, UK) containing 400µL 1x TE buffer.
2. The tube was heated at 80°C for 20 min in a waterbath to kill the cells and cooled at room temperature.
3. Fifty microlitres 10mg/mL lysozyme was added; following mixing by inversion and incubated overnight in a 37°C waterbath.
4. Seventy-five microlitres 10% SDS/proteinase K solution was added, tube inverted briefly and incubated for 10 min at 65°C.
5. One hundred microlitres 5M NaCl was added.
6. One hundred microlitres CTAB/NaCl (prewarmed in a 65°C waterbath); tube was mixed by inversion and incubated at 65°C for 10 min.
7. Seven hundred and fifty microlitres of chloroform (BDH, VWR International Ltd., Poole, England) was added; tube was inverted and centrifuged at 10,000 g for 5 min.
8. The aqueous supernatant was transferred to a sterile 2mL microfuge tube and 450µL ice cold isopropanol (BDH, VWR International Ltd., Poole, England) was added. The solution was mixed by gentle inversion.
9. The tube was placed at -20°C for 1hr or longer. Steps up to this point were carried out in containment level 3.

10. The tube was centrifuged at 10,000 *g* for 15 min.
11. The supernatant was removed and the pellet was washed with 1mL ice cold 70% ethanol and inverted gently.
12. The tube was centrifuged at 10,000 *g* for 5 min and the supernatant was discarded. The pellet was allowed to air dry for 30 min at a side angle.
13. The pellet size was estimated and resuspended overnight at 4°C overnight according to its size.

Pellet size	TE Buffer (μL)
none	20
small	50
medium	100
large	150

### 2.7.2 Crude DNA isolation

A single colony of *M. tuberculosis* was picked from a 7H10 agar plate and suspended into a sterile 2mL microcentrifuge tube containing 400μL of TE buffer. The sample was heat killed at 100°C for 20 min in an oven or waterbath. This is more than the standard heat kill of 80°C for 20 min (Doig et al. 2002). The supernatant was transferred into a clean microcentrifuge tube and stored at 4°C for subsequent PCR.

### 2.7.3 RNA extraction

1. RNA was extracted from each culture using the method of Mangan (Mangan, Monahan, & Butcher 2002) and the Q-Biogene Fast RNA Pro-Blue kit following the manufacturer's instructions (Q-Biogene Inc. CA, USA).
2. Four volumes of 5M GTC solution were added to each 50mL culture (section 2.2.11).
3. Each broth was spilt into universal tubes (approx 9-10) and harvested by centrifugation (Centaur 2, MSE, London, UK) at 2,000 g for 30 min.
4. The spent GTC was discarded and the pellets were pooled with the residual volume of GTC into 1 universal (~ 1mL).
5. To this aliquot, 1mL RNA Pro solution was added and the cells were resuspended by pipetting.
6. This suspension was transferred in 1mL aliquots to a Fast RNA Pro blue cap tube containing Lysing Matrix B and the tube was agitated in the ribolyser for 40 sec at a setting of 6.0.
7. The tube was centrifuged at 13,000 g for 5 min.
8. The supernatant was transferred to a new microfuge tube, taking care to avoid transferring the debris pellet and lysing matrix.
9. The sample was incubated at room temperature for 5 min to increase RNA yield.
10. Three hundred microlitres of chloroform was added and vortexed for 10 sec.
11. The tube was incubated for 5 min at room temperature to permit nucleoprotein dissociation and increase RNA purity.
12. The tube was centrifuged at 13,000 g for 5 min.

13. The upper phase was transferred to a new microcentrifuge tube.
14. Five hundred microlitres of ethanol was added, tube inverted 5 times to mix and then stored at -20°C overnight. Steps 1-14 were undertaken under containment level 3 conditions.
15. The following day, the tube was centrifuged at 13,000 g for 20 min at 4°C and then the supernatant removed.
16. The pellet was washed in 500µL of ice cold 70% ethanol, made with diethyl pyrocarbonate (DEPC) water.
17. Tube was centrifuged at 13,000 g for 15 min at 4°C and the ethanol was decanted.
18. The RNA pellet was air dried for 30 min on the bench.
19. The pellet was resuspended in 100µL of DEPC water. For subsequent time points at this stage, 2 tubes were pooled for each sample, so 50µL DEPC water added to each tube and combined.
20. The tubes were stored at -70°C or the RNA cleanup was performed immediately.

#### **2.7.4 RNA cleanup**

1. RNA samples were cleaned up using buffers from Qiagen RNeasy MiniElute Cleanup Kit for bacteria following manufacturer's instructions (Qiagen Ltd., West Sussex, UK).
2. All buffers were heated to 37°C for 30 min prior to clean up. Ten microlitres of β-mercaptoethanol was added per 1mL buffer RLT and 4 volumes of ethanol were added to Buffer RPE.

3. Three hundred and fifty microlitres of buffer RLT was added to the tube, followed by a further 250 $\mu$ L. The solution was mixed thoroughly by pipetting.
4. The sample (~ 700 $\mu$ L) was applied to an RNeasy mini column supported in a 2mL collection tube.
5. The tube was centrifuged for 15 sec at 10,000 g. The flow through was discarded along with the collection tube.
6. The RNeasy mini column was transferred to a new tube and 350  $\mu$ L of buffer RW1 was added. The tube was centrifuged at 10,000 g for 15 sec.
7. Ten microlitres of DNase stock solution was added to 70 $\mu$ L buffer RDD and mixed by gently inverting the tube.
8. The DNase:buffer RDD mix was added (80 $\mu$ L) directly onto the RNeasy silica gel membrane and left at room temperature for 15 min.
9. The column was washed through with 350 $\mu$ L buffer RW1 and centrifuged for 15 sec at 10,000 g.
10. The collection tube and flow through was discarded and the RNeasy column was transferred to a new 2mL collection tube.
11. Five hundred microlitres of buffer RPE was applied to the top of the column and centrifuged for 15 sec at 10,000 g.
12. A further 500 $\mu$ L buffer RPE was added to column and centrifuged for 2 min at 10,000 g.
13. The flow through was discarded and the tube was centrifuged for a further minute at 13,000 g to remove residual ethanol.
14. The column was transferred to a new collection tube and 30 $\mu$ L of RNase free water was pipetted directly onto the membrane to elute the RNA.

15. The tubes were incubated at room temperature for 5 min and centrifuged for 1 minute at 10,000 *g*.
16. Each tube was spilt into aliquots: 3µL (for RNA quantification and integrity evaluation using the Bioanalyser), 2 x 13µL for array hybridisations and stored at -70°C.

## 2.8 Polymerase chain reaction

### 2.8.1 Amplification of the gyrase A (*gyrA*) subunit of *M. fortuitum*

1. DNA was extracted from moxifloxacin resistant clonal populations; method was performed in containment level 2 facilities (section 2.7.1).
2. PCR was performed using previously described primers Pri 9 (5'-CGCCGCGTGCT[G,C]TATGC[A,G]ATG-3') and Pri 8 (5'-[C,T]GGTGG[A,G]TC[A,G]TT[A,G]CC[C,T]GGCGA-3') on each sample separately on three occasions to yield a 216bp product (Guillemin, Cambau, & Jarlier 1995). Ten microlitres of DNA was added to the PCR mix containing 10µL potassium chloride buffer 10x (Bioline Ltd., London, UK), 10µL of each primer Pri9 and Pri8 (4µM) (Sigma-Genosys Ltd., Haverhill, UK), 3µL deoxynucleoside triphosphates (dATP, dTTP, dGTP, dCTP; 2.5mM of each), 81.4µL PCR-quality water and 1µL of Taq polymerase (1 U) (Bioline). The amplification was performed on a Techgene thermal cycler (Techne, Princeton, NJ, USA). The program consisted of initial denaturation of 94°C for 5 min, then 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min and a final extension step of 72°C for 10 min.



3. PCR products were separated by gel electrophoresis on a 1.5% agarose gel. DNA bands were stained with ethidium bromide.
4. PCR products were cleaned up with QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA, USA) to remove primers and excess nucleotides. Cleaned up products were quantified on an agarose gel by comparison to HyperLadder I marker (Bioline, London, UK).
5. Sequencing was performed as described in section 2.8.6. Each sample was sequenced once in the forward direction and once in the reverse direction.
6. The sequences obtained from each sample were aligned with ClustalW (<http://www.ebi.ac.uk/clustalw/>) and compared to the wild type to identify base-pair mismatches.

### **2.8.2 Amplification of the RRDR of rifampicin resistant colonies**

1. Colonies were picked that had grown on 2µg/mL rifampicin and DNA was prepared as described in section 2.7.2
2. PCR was performed on the extracted DNA with 3 sets of primers designed to amplify 2 overlapping fragments of the *rpoB* (table 2.2).

**Table 2.2:** Primer sequences designed to amplify segments of *rpoB*. Set 1 and 2 from Jenkins et al 2005. Set 3 from Telenti et al 1993.

Primer Set	Amplicon Size (bp)	Primer Position	Primers
1	688	451-472 1119-1139	F 5' -TTC CCG ATG ATG ACC GAG AAG R 5' -GGA TCA GCT CGC CGA CCG TA
2	706	1029-1048 1714-1735	F 5' -CGA GGG TCA GAC CAC GAT G R 5' -GCG GGG CGA GAC GTC CAT GTA
3	411	1119-1138 1511-1530	F 5' -TAC GGT CGG CGA GCT GAT CC R 5' -TAC GGC GTT TCG ATG AAC C

3. Ten microlitres of DNA (40ng) was added to the PCR mix containing 81.4µL PCR-quality water, 10µL potassium chloride buffer (Bioline Ltd., London, UK) including 1.5mM magnesium chloride, 0.4µL of each primer (0.4µM) (Sigma-Genosys Ltd., Haverhill, UK), 3µL deoxynucleoside triphosphates (5mmol), and 1µL Taq (Bioline). The amplification was performed on a Techgene thermal cycler (Techne, Princeton, NJ, USA).
4. PCR products were separated by gel electrophoresis on a 1.5% agarose gel, and DNA bands were stained with ethidium bromide (section 2.8.3) and photographed (section 2.8.5).
5. Primers and excess nucleotides were removed from the amplified DNA with QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturers instructions.
6. The amount of DNA in the cleaned-up product was quantified by comparing the intensity of the band to bands of known intensity in a HyperLadder marker (Bioline).
7. Sequencing was performed as described in section 2.8.6.

### **2.8.3 Agarose Gels**

PCR products were electrophoresed through 1.5% agarose gels. Agarose powder (Bioline, London, UK) was weighed and melted into 0.5 X TBE buffer by heating until boiling in a microwave oven. Agarose was allowed to cool before ethidium bromide (Sigma Aldrich, Steinheim, Germany) was added to a final concentration of 0.5µg/mL. 5µL of 100bp ladder (Invitrogen, Paisley, UK) was loaded to each gel to size PCR amplicon bands. 3µL aliquot of loading buffer (0.25% bromophenol blue and 40% (w/v) sucrose in sterile distilled water) was mixed with 10µL of PCR product. This was loaded into wells and run at 110V for 30 min.

### **2.8.4 RNA quantification**

#### **2.8.4.1 Bioanalyser System**

RNA was quantified using the Agilent 2100 Bioanalyser system (Agilent Technologies Inc., Hanover, Germany) which also validates RNA integrity. The protocol was followed as per manufacturer's instructions. On some occasions when this was not possible, RNA was quantified using an agarose gel.

#### **2.8.4.2 Agarose Gel Electrophoresis**

1. A 1.2% agarose gel (Bioline, London, UK) was prepared in 1X TBE and 1.25µg/mL ethidium bromide (Sigma Aldrich, Steinheim, Germany).

2. One microlitre of RNA solution was run made up to a final volume of 10 $\mu$ L with RNase free water.
3. To each sample 4 $\mu$ L 6x loading buffer (Promega, Hampshire, UK) was added to make a final volume of 14 $\mu$ L.
4. Standards of *E. coli* rRNA (Sigma Aldrich, Steinheim, Germany) made up to concentrations of 0.02, 0.04, 0.08, 0.1 and 0.12 $\mu$ g/ $\mu$ L were run on the gel. Four microlitres of 6x loading buffer was added to 10 $\mu$ L of RNA standard solution, such that the total amount of RNA in each of the standards was 0.2, 0.4, 0.8, 1.0 and 1.2 $\mu$ g respectively.
5. The gel was run at 60V for approximately 45 min.

### **2.8.5 Gel Photography**

Gels were photographed with a digital camera DC120 Kodak Digital on a UV transilluminator (UVP Inc., California, USA) using camera software 2.0 (Kodak, California, USA). Images were stored as JPEG files.

### **2.8.6 Sequencing of samples**

Forward and reverse cycle sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Ready Reaction DNA sequencing kit (Applied Biosystems, Inc., Foster City, CA, USA). The reaction was performed with 40ng cleaned up DNA added to 3 $\mu$ L buffer, 3.2 $\mu$ L forward or reverse 1mmol primer, 1 $\mu$ L of cycle sequencing ready reaction mix and 10.8 $\mu$ L PCR-quality water.

The labelled DNA was precipitated by adding 62.5µL 95% ethanol (Royal Free Hospital Pharmacy), 3µL sodium acetate solution (2.3 mol/L) (Sigma Aldrich, Steinheim, Germany) and 14.5µL PCR-quality water and centrifuged at 13,000 g for 15 min at 4°C. The supernatant was removed with a fine tip pipette (Western Laboratory Services, Hampshire, UK), and the pellet was cleaned with 200µL 70% ethanol and then recentrifuged at 13,000 g for 15 min at 4°C. The supernatant was again removed and the pellet was dried at 37°C for 30 min. Four microlitres of formamide (Sigma Aldrich, Steinheim, Germany) and 1.5µL of sample were added to each well of the sequencing gel. The samples were then sequenced using a polyacrylamide sequencing gel (ABI 377 Applied Biosystems sequencer).

#### 2.8.7 Quantitative Reverse Transcriptase Real time PCR (qRT-PCR) Procedure

1. RNA was extracted as described in section 2.7.3
2. RT-PCR was performed using primers and probes in Table 2.3. The *dnaE2* probe was fluorescently labelled with 6-carboxyfluorescein (FAM) on the 5' end and Black Hole Quencher® I dye (Qiagen, Hilden, Germany) on the 3' end. The *sigA* probe was labelled with 6-carboxy-4', 5'-dichloro-2', 7'-dimethoxyfluorescein (JOE) on the 5' end and Black Hole Quencher® I dye (Qiagen, Hilden, Germany) on the 3' end.

**Table 2.3:** Probes and primers used for qRT-PCR

Target	Primer/Probe	Sequence	Reference
<i>dnaE2</i>	Probe	cagcgtcctgcaatgggacaaaga	Boshoff et al 2003
	Forward Primer	ccggtggaatgggcg	Boshoff et al 2003
	Reverse Primer	gcaatcggttggtgaaatt	Boshoff et al 2003
<i>sigA</i>	Probe	cctgcgcctggtggttcgc	Hampshire et al 2004
	Forward Primer	aaaccatctgctggaagcca	Hampshire et al 2004
	Reverse Primer	ttctcgacctgatccaggaag	This experiment

3. Five microlitres of RNA (approximately 1pg/reaction) was added to 1x QuantiTect Probe RT-PCR Master Mix (Qiagen, Hilden, Germany) including 4mM MgCl<sub>2</sub>, 0.4µM primers, 0.2µM probe, 0.5µL/reaction QuantiTect RT mix (Qiagen, Hilden, Germany) and made up to a final volume of 25µL with RNase-free water (Qiagen, Hilden, Germany).
4. The reaction was performed on a Rotorgene™ 3000 thermal cycler (Corbett Research, Mortlake, Australia) with the following conditions; a reverse transcription step 50°C for 30 min, PCR activation step 95°C for 15 min, and 45 repeats of 94°C for 15 sec and 60°C for 60 sec. Data was acquired during the combined annealing/extension step of 60°C on the FAM channel (excitation 470nm, detection 510nm, gain 10) or the JOE channel (excitation 530nm, detection 555nm, gain 10). The reaction components and the cycling conditions were all as outlined in manufacturer's instructions in the QuantiTect™ Probe RT-PCR Handbook.
5. Each sample was processed in triplicate, including a non-RT control for each sample also performed in triplicate.

6. All qRT-PCR experiments included a positive control of RNA from *M. tuberculosis* H37Rv.
7. The experiment was repeated.
8. The threshold level was set to 0.1, which was above the background fluorescence of the no template control in the exponential phase of the curve. The crossing cycle threshold value (Ct) was the cycle at which there was a significant increase in fluorescence above the specified threshold. The data was analysed using comparative Ct's. Delta ( $\Delta$ ) Ct's are calculated as the difference between the Ct with RT treatment and without. Then the relative gene expression was calculated using the equation  $2^{-\Delta\Delta Ct}$ , where  $\Delta\Delta Ct$  is the difference between the  $\Delta Ct$  for the tested gene *dnaE2* from the  $\Delta Ct$  of the housekeeping gene, *sigA* (Livak & Schmittgen 2001). The efficiency of each probe and primer pair was greater than 99%.

## 2.9 BμG@S Microarray Protocol

### 2.9.1 RNA versus DNA: Labelling and Hybridisation

*M. tuberculosis* whole genome arrays were supplied by the Wellcome Trust BμG@S group at St George's, University of London. The array consisted of PCR products (11552 features) printed onto GAPSII glass slides representing 4410 genes from H37Rv and CDC1551 *M. tuberculosis* and *M. bovis* genomes.

All reagents are supplied by Invitrogen Life Technologies, except for Bovine Serum Albumin (BSA) Fraction V 96-99% (100mg/mL), 20x Salt Sodium Citrate (SSC),

Sodium Dodecyl Sulphate (SDS) which are supplied by Sigma Aldrich, MinElute PCR Purification Kit supplied by Qiagen, 22x22mm LifterSlips VWR International, Microarray Hybridisation Cassette by Corning, staining troughs and racks by Raymond A. Lamb and Coplin staining jar by Fisher Scientific.

### 2.9.2 Cy5 label DNA

H37Rv genomic DNA was used to prepare Cy5-dCTP labelled cDNA as the comparator. One Cy5 labelled DNA sample was prepared per microarray slide.

Each sample: DNA	20 $\mu$ L (1 $\mu$ g)
Random primers	1 $\mu$ L (3 $\mu$ g/ $\mu$ L)
H <sub>2</sub> O	to 41.5 $\mu$ L

The mix was heated at 95°C for 5 min, snapped cooled on ice and briefly centrifuged.

To each tube the following reagents were added.

Reagents	Volume ( $\mu$ L)
10x React 2 buffer	5
dNTPs (5mM aA/G/TTP, 2mM dCTP)	1
Cy5 dCTP	1.5
DNA Polymerase I Large (Klenow) Fragment (3-9U/ $\mu$ L)	1

The tube was incubated at 37°C in the dark for 90 min.



### 2.9.3 Cy3 label RNA

Aliquots of approximately 2 to 10 $\mu$ g (equivalent to approximately 2-5  $\times 10^8$  bacteria) of total RNA were used as a template for cDNA synthesis and fluorescent dye analogues incorporated during reverse transcription using Cy3-dCTP. One Cy3 labelled RNA sample was prepared per microarray slide.

Each sample: RNA	2-10 $\mu$ g
Random primers	1 $\mu$ L
H <sub>2</sub> O	to 11 $\mu$ L

The mix was heated at 95°C for 5 min, snapped cooled on ice and briefly centrifuged.

To each tube the following reagents were added.

Reagents	Volume ( $\mu$ L)
5x First Strand buffer	5
Dithiothreitol (DTT) (100mM)	2.5
dNTPs (5mM aA/G/TTP, 2mM dCTP)	2.3
Cy3 dCTP	1.7
Superscript II Reverse Transcriptase (200U/ $\mu$ L)	2.5

The tube was incubated at 25°C in the dark for 10 minutes, followed by 42°C in dark for 90 min.

### 2.9.4 Prehybridise slide

#### Prehybridisation solution

Reagents	Volume (mL)	Final Concentration
20xSSC	8.75	3.5xSSC
20% SDS	0.25	0.1% SDS
BSA	5	10mg/mL
H <sub>2</sub> O	36	

The prehybridisation solution was mixed in a Coplin jar and incubated at 65°C during the labelling reaction to equilibrate. The microarray slide was incubated in the pre-heated prehybridisation solution at 65°C for 20 min. The slide was rinsed thoroughly in 400mL H<sub>2</sub>O for 1min followed by a rinse in 400mL propan-2-ol for 1 min. The slide was centrifuged in 50mL falcon tube at 1000 g for 5 min to dry with label side down. The slide was stored in a dark, dust-free box until hybridisation (<1hr).

### 2.9.5 Qiagen MinElute Purification to purify Cy3/Cy5 labelled cDNA/DNA

Cy3 and Cy5 labelled cDNA samples were combined in a single tube and 375µL Buffer PB was added. The sample was applied to the MinElute column in a collection tube and centrifuged at 13,000 g for 1 min. The flow through was discarded and the MinElute column was placed back into the same collection tube. Five hundred µL Buffer PE was added to the MinElute column and centrifuged at 13,000 g for 1 min. The flow-through was discarded and the MinElute column was placed back into the same collection tube. Two hundred and fifty microlitres Buffer PE was added to the

MinElute column and centrifuged at 13,000 *g* for 1 min. The flow-through was discarded and the MinElute column was placed back into the same collection tube. The tube was centrifuged for an additional 1 min to remove residual ethanol. The MinElute column was placed into a fresh 1.5mL micro-centrifuge tube. To the centre of the membrane 31.8µL H<sub>2</sub>O was added and left to stand for 1 min. Volume used was determined by the hybridisation volume required for different sizes of LifterSlips (in this case two 22x22mm LifterSlips are used). The tube was centrifuged at 13,000 *g* for 1 min.

### 2.9.6 Wash Preparation

#### Wash A

Reagents	Volume (mL)	Final Concentration
20xSSC	20	1xSSC
20% SDS	1	0.05% SDS
H <sub>2</sub> O	to 400	

Wash A was placed in a sealed bottle and incubated at 65°C along with an empty staining trough overnight.

### 2.9.7 Hybridise slide

The freshly pre-hybridised microarray slide was placed in a hybridisation cassette and two 15µL aliquots of H<sub>2</sub>O were added to the wells in the cassette. The purified Cy3/Cy5 labelled cDNA/DNA sample was mixed with the hybridisation solution.

## Hybridisation Solution

Reagents	Volume ( $\mu\text{L}$ )	Final Conc
Cy3/Cy5 labelled cDNA/DNA sample	29.8	
Filtered 20x SSC	9.2	4x SSC
Filtered 2% SDS	7	0.3% SDS

The hybridisation solution was heated at 95°C for 2 min. The solution was allowed to cool slightly and briefly centrifuged. The LifterSlips were placed carefully over the arrayed area of the slide, ensuring that the LifterSlip Bars were face down with a small gap in between both LifterSlips and that the array surface was not scratched. Half of hybridisation solution was carefully pipetted under one corner of the LifterSlip, allowing the solution to be drawn completely across the array by capillary action. The other half of the hybridisation solution was pipetted under the opposite corner of the other LifterSlip with any excess being pipetted in the gap between the two LifterSlips. The hybridisation cassette was submerged in a water bath at 65°C in the dark for 16-20hr.

### 2.9.8 Wash slide

The pre-heated Wash A at 65°C was added to the pre-heated staining trough at 65°C. The microarray slide was removed from the hybridisation cassette and the slide was washed initially by carefully placing in staining trough of Wash A at 65°C to remove the LifterSlip. Once the LifterSlip was displaced, the slide was placed in a slide rack and agitated for a further 2 min.

The slide was transferred to a clean slide rack and agitated in first trough of Wash B for 2 min. The slide was washed in 2 subsequent troughs of Wash B with agitation for 2 min.

#### **Wash B**

<b>Reagents</b>	<b>Volume (mL)</b>	<b>Final Concentration</b>
20xSSC	1.2	0.06xSSC
H <sub>2</sub> O	to 400	

The slide was placed into a 50mL falcon tube and centrifuged at 1,000 g for 5 min to dry slide.

#### **2.9.9 Scan slide**

Arrays were scanned using an Affymetrix™ 428 array scanner (Affymetrix Inc, Santa Clara, USA). The slide was scanned at 3 different gain values with the Cy5 channel first, followed by 3 scans with the Cy3 channel.

#### **2.9.10 Data Analysis**

For analysis using ImaGene™ software, 3 scans were saved in each channel, one below saturation, one +10 gain, one +20 gain. All images were overlaid for analysis in ImaGene™ and then put through MAVI program before entry into GeneSpring™. Bluefuse™ analysis was performed on the scans which were just below spot saturation before entry into GeneSpring™. Data acquisition and analysis was performed using GeneSpring™ software.

Data normalisation: Values below 0.01 were set to 0.01. Each gene's measured intensity was divided by its control channel value in each sample; if the control channel was below 10 then 10 was used instead. If the control channel and the signal channel were both below 10 then no data was reported. Each measurement was divided by the 50<sup>th</sup> percentile of all measurements in that sample. The percentile was calculated using only genes marked present or marginal. Specific samples were normalized to one another: all samples were normalized against the mean of the control samples. Each measurement for each gene in those specific samples was divided by the mean of that gene's measurements in the corresponding control samples. If all of the control measurements were flagged absent or unknown then no data was reported.

## Chapter 3: Heteroresistance

### 3.1 Introduction

Heteroresistance is the simultaneous occurrence of sub-populations of resistant and sensitive bacteria within the same clinical sample (section 1.12).

#### 3.1.1 *M. fortuitum* as a Model System of *M. tuberculosis*

*M. fortuitum* is used as a model of *M. tuberculosis* in this chapter. It is a useful *in vitro* model system because it is fast growing unlike the slow growing *M. tuberculosis* and it does not require containment level 3 facilities. Other studies have used *M. fortuitum* as a model for *M. tuberculosis* in structure activity relationships, mycobacterial susceptibility testing and mutant generation (Gillespie, Morrissey, & Everett 2001; Gillespie et al. 2005; Renau et al. 1995).

#### 3.1.2 Aim of chapter

The aim of this chapter is to investigate phenotypic heteroresistance in *M. fortuitum* and *M. smegmatis*. Further investigation into this phenomenon may lead to an explanation of patient treatment failures and to the discovery of new targets for tuberculosis treatment. It may also serve as a mechanism to predict development of homogenous resistance in a given patient. Besides demonstrating mixed populations of resistant and sensitive bacteria in a single isolate we also suggest that

heteroresistance is a phenotypic phenomenon which is expressed depending on the physiological state of the bacteria.

### **3.2 Materials and methods**

The MIC was determined using methods described in section 2.4. Resistant daughter clonal populations were isolated and the MIC was determined (section 2.4.3). A region encompassing the QRDR of *gyrA* was screened for mutations (section 2.8.1).

### **3.3 Results**

#### **3.3.1 Experimental Assumptions**

Identification of sensitive and resistant sub-populations of bacteria in one isolate suggests the presence of heteroresistance. It is assumed that a single colony represents a clonal population. It is assumed that the definition of MIC in this experiment is the lowest concentration of anti-microbial which inhibited the greatest number of colonies. For clonal populations the MIC was assumed to be the lowest concentration of anti-microbial that inhibited growth of the population. This is termed a clonal population MIC as the colony was cultivated before MIC determination. The progeny from a single colony was defined as resistant when it grew at twice the MIC of that strain.



### 3.3.2 MIC Distribution of Control Strains

*M. fortuitum* NCTC 10394 was used as a control to represent a laboratory adapted strain. The MIC for this strain was 0.06µg/mL moxifloxacin with the growth of 28/34 clonal populations inhibited at this concentration (table 3.1). This MIC value was confirmed using method 2.4.1. Four clonal populations had a MIC of 0.12µg/mL (2 X MIC) and 2 clonal populations had a MIC of 0.25µg/mL (4 X MIC). Both populations were determined to be resistant as their MIC was at least twice the MIC for that strain.

*M. smegmatis* NCTC 8159 and *M. smegmatis* mc<sup>2</sup>155 were tested to determine whether the presence of sensitive and resistant organisms in the same culture could be identified in a different mycobacterial species. The genome sequencing of this species is nearly completed so it would facilitate investigation into any genetic mechanism of heteroresistance. The MIC of *M. smegmatis* mc<sup>2</sup>155 was 0.1µg/mL moxifloxacin for 40/42 clonal populations, 0.1µg/mL minocycline for 38/42 clonal populations and 0.5µg/mL amikacin for 38/42 clonal populations (table 3.2). All bacterial growth of this strain was prevented at 0.2µg/mL moxifloxacin, 0.4µg/mL minocycline and 1.5µg/mL amikacin. *M. smegmatis* NCTC 8159 had a MIC range of 0.1-0.2µg/mL moxifloxacin for 83/84 clonal populations, 1 clonal population had a MIC of 0.3µg/mL. For minocycline, 41/42 clonal populations had a MIC of 0.2µg/mL, 1 clonal population had a MIC of 0.3µg/mL. For amikacin 41/42 clonal populations had a MIC of 0.5µg/mL and 1 clonal population had a MIC of 1.5µg/mL. It was not possible to get a clinical isolate of *M. smegmatis*.

### 3.3.3 MIC Distribution of Clinical Isolates

#### 3.3.3.1 Experimental Replicates

For isolate RF 01:332, experiments were performed on 3 occasions for the drugs moxifloxacin and minocycline and on 4 occasions for the drug amikacin (table 2.1). For isolates RF 02:159 and RF 02:173 single experiments were performed as both isolates yielded similar results. The MIC values were recorded from these experiments.

#### 3.3.3.2 MIC's of *M. fortuitum* RF 01:332

Distribution of MIC values for isolate RF 01:332 showed resistant clonal populations to all three drugs. The strain MIC varied for each experiment (table 3.1). It was 0.2µg/mL (25/42 clonal populations), 0.2 - 0.3µg/mL (40/42 clonal populations) and 0.1µg/mL (18/42 clonal populations) moxifloxacin in experiment 1, 2 and 3 respectively. In experiment 1, there were 8 clonal populations with a MIC of 0.5µg/mL moxifloxacin which is 2.5 times the MIC for this isolate, 2 clonal populations with a MIC of 0.7µg/mL and 2 with a MIC of >2µg/mL (clonal populations grew at 2µg/mL), which is > 10 times the MIC (figure 3.1). No resistant clonal populations were isolated in experiment 2, perhaps because of a higher strain MIC (0.2 - 0.3µg/mL). In experiment 3, 10 clonal populations had a MIC of 0.2µg/mL, twice the strain MIC for this experiment (0.1µg/mL), 12 clonal populations had a MIC of 0.3µg/mL and 2 clonal populations had a MIC of 0.9µg/mL. The strain MIC of minocycline for *M. fortuitum* RF 01:332 was 0.4µg/mL (30/42 clonal

populations), 0.3µg/mL (18/42 clonal populations) and 0.2µg/mL (34/42 clonal populations) in experiment 1, 2 and 3 respectively (table 3.1). In experiment 1, no clonal populations were resistant (grew at > twice the strain MIC). In experiment 2, 2 clonal populations were resistant with a MIC of 1µg/mL (3.5 X MIC). The MIC of amikacin for *M. fortuitum* RF 01:332 was 1µg/mL in experiment 1 and 0.5µg/mL in subsequent experiments. In experiment 1, no resistant clonal populations were isolated. In experiment 2, 7 clonal populations were resistant at 1µg/mL amikacin (2 X MIC). In experiment three, 14, 2 and 2 clonal populations had a MIC of 1, 1.5 and 3µg/mL amikacin respectively, all deemed to be resistant as were 2 X or more than the MIC of 0.5µg/mL. In experiment four, 4 clonal populations were resistant with a MIC of 1µg/mL amikacin.

### 3.3.3.3 MIC's of *M. fortuitum* RF 02:159 and RF 02:173

No moxifloxacin resistant clonal populations were isolated from clinical strain *M. fortuitum* RF 02:159 (figure 3.1). Four, 15 and 23 clonal populations of *M. fortuitum* RF 02:159 had MIC's of 0.1, 0.2 and 0.3µg/mL moxifloxacin respectively in experiment 1. Forty-two clonal populations had a MIC of 0.2µg/mL in experiment 2. Eight, 24 and 10 clonal populations had MIC's of 0.1, 0.2 and 0.3µg/mL respectively in experiment 3. One minocycline (MIC of 0.5µg/mL) and 9 amikacin (MIC of 1µg/mL) resistant clonal populations were isolated. Forty one clonal populations of *M. fortuitum* RF 02:159 had a MIC of 0.25µg/mL minocycline. Thirty-three clonal populations of *M. fortuitum* RF 02:159 were inhibited at 0.5µg/mL amikacin. No moxifloxacin resistant clonal populations were isolated from the clinical strain *M. fortuitum* RF 02:173 isolated from the same patient. Thirteen clonal populations had a

MIC of 0.1µg/mL and 29 clonal populations had a MIC of 0.2µg/mL. Seventeen minocycline-resistant clonal populations were isolated with a MIC of 0.25µg/mL and 25 clonal populations had a MIC of 0.12µg/mL (sensitive). Twenty five clonal populations were inhibited at 0.5µg/mL amikacin and 10 were inhibited at 1µg/mL.

### 3.3.4 Reversion of Resistance

Clonal population 12, a resistant daughter colony of *M. fortuitum* RF 01:332 was isolated at 2µg/mL moxifloxacin and also had a MIC of 0.7µg/mL minocycline (table 3.3). It was found that against moxifloxacin and minocycline, the previous population of resistant colonies had disappeared, as the colonies from this isolate had MIC's of 0.2µg/mL moxifloxacin and 0.1-0.3µg/mL minocycline. The phenotype of the resistant daughter colony was not retained. In experiment 1, 14 clonal populations had a MIC of 0.1µg/mL and 28 clonal populations had a MIC of 0.2µg/mL. No moxifloxacin-resistant clonal populations were detected. In experiment 2, 15/42 clonal populations had a MIC of 0.2µg/mL, 13 were resistant with a MIC of 0.4µg/mL moxifloxacin (2 X MIC). When tested against minocycline, 40/42 clonal populations of *M. fortuitum* RF 01:332 clonal population 12 had a MIC of 0.1µg/mL. The other 2 clonal populations were resistant with a MIC of 0.2µg/mL (2 X MIC). In the repeat experiment, 14, 17 and 11 clonal populations had MIC's of 0.2, 0.3 and 0.4µg/mL minocycline respectively. The incubation period for these plates exceeded previous incubation steps. The experiment was repeated with clonal population 15 *M. fortuitum* RF 01:332 which had a MIC of 0.8µg/mL minocycline but was completely sensitive.

### 3.3.5 Screening of the QRDR of *gyrA* of resistant colonies

The analysis of the QRDR of *gyrA* from 3 moxifloxacin resistant colonies of *M. fortuitum* RF 01:332 showed no mutations (figure 3.2). The 3 clonal populations had a MIC 0.7, 0.9 and > 2 µg/mL moxifloxacin. Genotypic analysis of minocycline and amikacin resistant colonies were not possible due to multiple mechanisms of resistance acquisition.

**Table 3.1:** The variation in the determined MIC values for each isolate to moxifloxacin, minocycline and amikacin.

Species	Isolate	MIC (µg/mL)					
		Moxifloxacin	Exp	Minocycline	Exp	Amikacin	Exp
<i>M. smegmatis</i>	NCTC 8159	0.1	1	0.2	1	0.5	1
		0.2	2	ND		ND	
	mc <sup>2</sup> 155	0.1	1	0.1	1	0.5	1
<i>M. fortuitum</i>	NCTC 10394	0.06	1	ND		ND	
	RF 01:332	0.2	1	0.4	1	1	1
		0.2-0.3	2	0.3	2	0.5	2
		0.1	3	0.2	3	0.5	3
		ND		ND		0.5	4
	RF 02:159	0.3	1	0.25	1	0.5	1
		0.2	2	ND		ND	
		0.2	3	ND		ND	
	RF 02:173	0.2		0.12	1	0.5	1

ND not determined

**Table 3.2:** The proportion of clonal populations that are resistant to each drug ( $\geq 2 \times$  MIC) for each strain and which strains were heteroresistant as determined by each drug.

Species	Isolate	No. of resistant clonal populations/total no. of clonal populations (%)					
		Moxifloxacin	H	Minocycline	H	Amikacin	H
<i>M. smegmatis</i>	mc <sup>2</sup> 155	2/42 (4.8)	√	4/42 (9.5)	√	4/42 (9.5)	√
	NCTC 8159	1/84 (1.2)	√	0/42	×	1/42 (2.4)	√
<i>M. fortuitum</i>	NCTC 10394	6/34 (17.6)	√	ND		ND	
	RF 01:332	36/126 (28.6)	√	4/126 (3.2)	√	29/168 (17.3)	√
	RF 02:159	0/126	×	1/42 (2.4)	√	9/42 (21.4)	√
	RF 02:173	0/42	×	17/42 (40.5)	√	10/35 (28.6)	√

H – heteroresistant

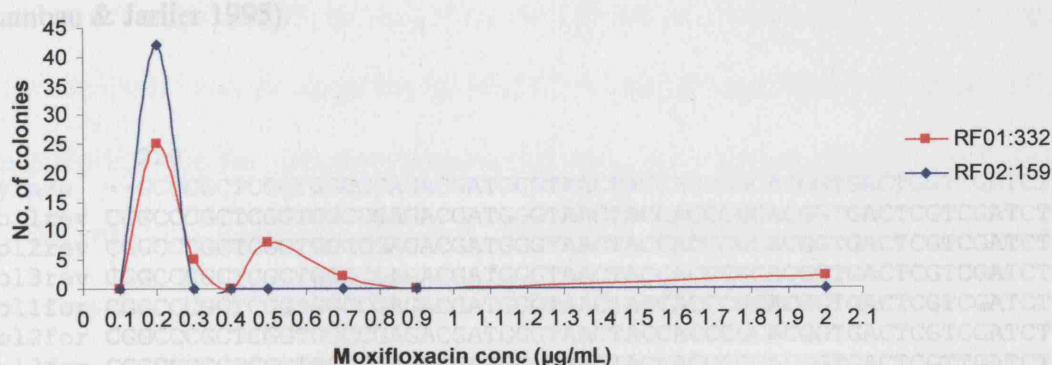
ND – not determined

**Table 3.3:** Results for clonal population 12, a first generation moxifloxacin mutant of *M. fortuitum* RF 01:332 which had a MIC of  $> 2 \mu\text{g/mL}$  moxifloxacin and  $0.7 \mu\text{g/mL}$  minocycline which was then re-tested.

Drug	MIC ( $\mu\text{g/mL}$ )		No. of resistant clonal populations/total no. of clonal	Hetero-resistant
	Exp 1	Exp 2		
Moxifloxacin	0.2	0.2	13/84 (15.5)	√
Minocycline	0.1	0.3	2/84 (2.38)	√

**Figure 3.1:** An illustrative example of a strain with normal MIC pattern, *M. fortuitum* RF 02:159, and a strain with a heteroresistant pattern, *M. fortuitum* RF 01:332 (for illustrative purposes). Clonal populations were isolated depending on their MIC to moxifloxacin. Similar patterns were observed with all other strains depending on whether they were heteroresistant (data not shown).

Individual colony MIC values for *M. fortuitum* RF01:332 and RF02:159



Cul = colony

**Figure 3.2:** Sequencing of the QRDR of *M. fortuitum* *gyrA*. A multiple sequence alignment of 3 moxifloxacin resistant clonal populations isolated from *M. fortuitum* RF 01:332 (performed in Clustal W 1.82). No mutations were found within the 120bp QRDR (bases 220-339 *M. tuberculosis gyrA* Accession number AJ564396). A consensus sequence was taken from 2 separate sequence reactions in both directions and from 3 different PCR reactions. When it was Blast searched, there was a 100% identity with *M. fortuitum* partial *gyrA* (Accession number X87122, Guillemin, Cambau & Jarlier 1995).

```

                220
gyrase  --GCCCCGCTCGGTGGCCGAGACGATGGGTAACCTACCACCCGCACGGTGACTCGTCGATCT
collrev  CGCCCCGCTCGGTGGCCGAGACGATGGGTAACCTACCACCCGCACGGTGACTCGTCGATCT
col2rev  CGCCCCGCTCGGTGGCCGAGACGATGGGTAACCTACCACCCGCACGGTGACTCGTCGATCT
col3rev  CGCCCCGCTCGGTGGCCGAGACGATGGGTAACCTACCACCCGCACGGTGACTCGTCGATCT
collfor  CGCCCCGCTCGGTGGCCGAGACGATGGGTAACCTACCACCCGCACGGTGACTCGTCGATCT
col2for  CGCCCCGCTCGGTGGCCGAGACGATGGGTAACCTACCACCCGCACGGTGACTCGTCGATCT
col3for  CGCCCCGCTCGGTGGCCGAGACGATGGGTAACCTACCACCCGCACGGTGACTCGTTGATCT
                *****

                339
gyrase  ACGACACCCTGGTCCGGATGGCCCAGCCGTGGTCACTGCGCTATCCGCTGGTTCGACGGTC
collrev  ACGACACCCTGGTCCGGATGGCCCAGCCGTGGTCACTGCGCTATCCGCTGGTTCGACGGAC
col2rev  ACGACACCCTGGTCCGGATGGCCCAGCCGTGGTCACTGCGCTATCCGCTGGTTCGACGGAC
col3rev  ACGACACCCTGGTCCGGATGGCCCAGCCGTGGTCACTGCGCTATCCGCTGGTTCGACGGAC
collfor  ACGACACCCTGGTCCGGATGG-----
col2for  ACGACACCCTGGTCCGGATGGCCCAGCCGTGGTCACTGCGCTATT-----
col3for  ACGACACCCTGGTCCGGATGGCCCAGCCGTGGTCACTGCGACTAT-----
                *****

```

Col = colony

### 3.4.2 Heteroresistance in *M. fortuitum* Clinical Isolates

Phenotypic heteroresistance to moxifloxacin, minocycline and amikacin in *M. fortuitum* RF 01:332 was demonstrated (table 3.2). This isolate is multi-drug resistant, although the molecular basis is unknown. Resistant clonal populations were isolated at 2µg/ml moxifloxacin showing growth at 10 times the MIC value. The inoculum used in this experiment was  $10^5$  organisms ( $10^2$  per ml) so you would expect <1



### 3.4 Discussion

#### 3.4.1 Experimental Findings

The demonstration that a sub-population of antibiotic resistant organisms can exist in an otherwise sensitive population of organisms causing infection is termed heteroresistance. This study has demonstrated this phenomenon in laboratory strains *M. smegmatis* NCTC 8159, *M. smegmatis* mc<sup>2</sup>155 and *M. fortuitum* NCTC 10394 and in the clinical isolates *M. fortuitum* RF 01:332, RF 02:159 and RF 02:173 (table 3.2). These findings are the first demonstration of phenotypic heteroresistance in clinical isolates of *M. fortuitum* and in laboratory maintained strains, although heteroresistance has previously been demonstrated in clinical isolates of *M. tuberculosis* (Rinder, Mieskes, & Loscher 2001). The incidence of heteroresistance must be considered in a clinical setting as it may result in resistance to a drug becoming fixed in a population of bacteria at an increased frequency. This would have serious implications for both the risk of progression to phenotypic resistance and the interpretation of rapid susceptibility testing methods using molecular techniques.

#### 3.4.2 Heteroresistance in *M. fortuitum* Clinical Isolates

Phenotypic heteroresistance to moxifloxacin, minocycline and amikacin in *M. fortuitum* RF 01:332 was demonstrated (table 3.2). This isolate is multi-drug resistant, although the molecular basis is unknown. Resistant clonal populations were isolated at 2µg/mL moxifloxacin showing growth at 10 times the MIC value. The inoculum used in this experiment was 10<sup>5</sup> organisms (10<sup>8</sup> per mL) so you would expect <1

spontaneous mutations per experiment (no growth above the MIC). The observation of resistant clonal populations in this experiment is greater than can be explained by the intrinsic mutation rate (about  $10^{-7}$  -  $10^{-9}$  per gene per cell division). Resistant clonal populations with MIC's of 0.4, 0.5, 0.7 and 0.9 µg/mL moxifloxacin were also observed. When selected on minocycline, 4/126 clonal populations were resistant with MIC's of 0.5 and 1 µg/mL, more than twice the MIC of that strain. There was a large portion of clonal populations resistant to amikacin with 29/168 having a MIC greater than twice the strain MIC. Growth above the MIC was reproducible for this isolate to all three compounds, reducing the probability that the clonal populations were isolated by chance. The demonstration of heteroresistance to all three compounds indicates that mutant selection occurs regardless of which antibiotic is used in selection.

Phenotypic heteroresistance was demonstrated in clinical isolates *M. fortuitum* RF 02:159 and RF 02:173, which are from the same patient as RF 01:332 isolated 12 months later, to minocycline and amikacin (table 3.2). The disappearance of moxifloxacin-resistant colonies might be due to the patient receiving treatment for a *M. fortuitum* infection in this 12 month period (patient notes were unavailable). It is unlikely that loss of heteroresistance to moxifloxacin in this isolate is as a result of antibiotic pressure as such would have been expected to result in an increased MIC result. Also moxifloxacin was not available commercially in 2002 so it was not used in treatment but perhaps another quinolone was. Another likely explanation is that the phenomenon of heteroresistance is due to differential gene expression which may change over time and it just depends on the physiological state of the bacteria at the time of investigation.

### 3.4.3 Heteroresistance in Laboratory Strains

Phenotypic heteroresistance was observed in the laboratory strains *M. smegmatis* mc<sup>2</sup>155 to all three drugs and *M. smegmatis* NCTC 8159 to moxifloxacin and amikacin (table 3.2). These are wild-type strains which are less likely to possess multiple chromosomal mutations by chance so the demonstration of heteroresistance in these strains is more likely attributed to non-genetic mechanisms. The organism *M. smegmatis* has an advantage over *M. fortuitum* for use as a model system in that there is a near completion sequence database, currently being developed by TIGR which would enable investigation into any genetic determinants of a heteroresistant phenotype. However no clinical isolates of *M. smegmatis* were investigated as they are rare and not routinely isolated in a clinical setting as it is a commensal organism. This finding of phenotypic heteroresistance in laboratory isolates was confirmed in *M. fortuitum* NCTC 10394 to moxifloxacin where 6/34 clonal populations were resistant (table 3.2). The discovery of heteroresistance in laboratory strains and in clinical isolates demonstrates that it is possible that mixed populations of sensitive and resistant bacteria can be observed in any environment regardless of whether it is maintained in the laboratory or from the complex environment of a patient.

### 3.4.4 Sub-population of Resistant Bacteria

It is intriguing that a heterogeneous population of bacteria can occur prior to drug exposure. It is possible that every bacterial isolate is predisposed to having a sub-population of intermediately resistant bacteria which are resistant to some drugs but not to others, leading to growth of colonies on drug concentrations above the MIC.

This sub-population may decline during therapy as a result of exposure to drugs which the isolate is sensitive to. Another explanation is that the length of time the clinical isolate is maintained *in vitro* might be significant. This may permit the population of initially sensitive bacteria to develop resistance to certain drugs and not to others. However this is not the case in this experiment as the isolates were stored long term at -70°C which should have prevented any physiological change among the bacteria. Also other studies have demonstrated heteroresistance using different methods of maintaining the clinical isolates *in vivo* (Mondon et al. 1999; Ryffel et al. 1994).

#### 3.4.5 Reversion of Resistant Clonal populations

The MIC's of the resistant daughter clonal populations of *M. fortuitum* RF 01:332 were investigated to see if they maintained their resistant phenotype. Results showed that these clonal populations revert back to having low MIC's of 0.2µg/mL moxifloxacin and 0.3µg/mL minocycline from growing at 2µg/mL moxifloxacin and having a MIC of 0.7µg/mL minocycline in the previous experiment. In this case repeating the MIC determination did not result in the emergence of a high resistance phenotype. These results may suggest heteroresistance is as a result of the expression of certain genes rather than a genetic mutation and that this heteroresistant phenotype may also be a result of bacteria being in different physiological state.

#### 3.4.6 Genetic Determinants of Heteroresistance

To further investigate whether heteroresistance was as a result of a genetic mutation, the QRDR of the *gyrA* of moxifloxacin-resistant clonal populations were amplified

and no mutations were revealed even in the clonal population which had a MIC of > 2µg/mL moxifloxacin (figure 3.2). Moxifloxacin disrupts DNA supercoiling and resistance to this quinolone develops due to mutations in the gyrase genes, *gyrA* and *gyrB* which encode the enzyme DNA topoisomerase II (Guillemin, Jarlier, & Cambau 1998) (section 1.11.4.1). Low level quinolone resistance may be conferred by non-gyrase mutations (Zhou et al. 2000) but in this case resistant clonal populations analysed were four and ten times the MIC. A variety of *gyrA* alleles can occur causing a variation in clonal population MIC's among a mutant mycobacterial population. This was not observed in the genetic analysis in this experiment but there was variation in the MIC's of individual bacilli which may possess changes in different resistance alleles. This was not seen in this experiment but perhaps this would have been possible if a greater number of mutants were analysed, along with the investigation of *gyrB* mutations. No mutation in *gyrA* corroborates the previous conclusion that heteroresistance observed in this setting is a phenotypic phenomenon and indicates that the presence of the selecting antibiotic was not required for the generation of resistant mutants. The genetic mechanism behind heteroresistance could only be investigated in moxifloxacin resistant colonies as resistance to minocycline and amikacin occurs due to complex efflux pump mechanisms and variations in cell wall permeability. Both amikacin (an aminoglycoside) and minocycline (a tetracycline analogue) interfere with protein synthesis. Efflux pumps, LfrA protein of *M. smegmatis*, play a role in low level resistance to fluoroquinolone and other compounds (Liu, Takiff, & Nikaido 1996; Takiff et al. 1996). Tetracycline resistance mediated by tap proteins has been identified in *M. fortuitum* (Ainsa et al. 1998). Further work is required to confirm whether heteroresistance is determined by the physiological environment or by a genetic determinant.

### 3.4.7 Heteroresistance is not restricted to a particular drug

An interesting observation was that clonal populations which were identified as having high MIC's against one drug, were likely to have high MIC's to another drug. For instance, clonal population 12 of *M. fortuitum* RF 01:332 had a MIC of  $> 2\mu\text{g/mL}$  moxifloxacin and  $0.7\mu\text{g/mL}$  minocycline. This confirms the suggestion that heteroresistance is not restricted to a particular drug in agreement with Rinder et al and is as a result of a general genetic expression mechanism. If this is the case, then there is a concern that all clinical isolates at some stage have populations of bacteria that are resistant to antibiotics which act on different physiological mechanisms. Also it may be possible that the population of bacteria which are resistant to one drug are cross resistant to other drugs and this would support the hypothesis that it is expression of certain genes rather than a mutation which leads a strain to be heteroresistant.

### 3.4.8 Clinical Significance of Heteroresistance

The clinical significance of heteroresistance remains to be determined. It is likely that the ability to detect mixed populations of bacteria in a patient isolate would improve treatment, which may reduce the risk of progression to homogenous resistance. Though it is not known if there is a level of heteroresistance above which it is necessary to alter the patient's therapy due to the risk of developing homogenous resistance and whether this can be detected by molecular mechanisms. The resistant sub-population may disappear under different physiological conditions due to change

in the expression of certain genes, thus the detection of this resistance may be meaningless. However mutants from this resistant population may be selected by fluctuations of antibiotic concentrations which occur during treatment which has serious implications for therapy. This re-emphasises the importance of treatment with multiple agents for mycobacterial infections to prevent treatment failure due to the emergence of resistance.

#### 3.4.9 Experimental limitations and future work

The approach used in this experiment was to test the MIC's of clonal populations rather than individual colony MIC's as the colonies were allowed to multiply in broth in order to get sufficient numbers to get a MIC. This procedure makes the assumption that the bacterial population from a single colony is clonal. The culturing step could be avoided by picking a single colony and inoculating it into broth and then immediately performing the determination of the MIC's using the multi-inoculator system. Furthermore in the investigation into the genetic determinants of moxifloxacin resistance, the QRDR of *gyrB* could be investigated.

It would be interesting to adapt the heteroresistance experiments so that the phenomenon could be confirmed in *M. tuberculosis* and to perform further genetic screening to determine if there are any genes associated with the phenomenon. A mutation in *mutT* has occurred in the Beijing strain of *M. tuberculosis* which is reported to correlate with drug resistance (Rad et al. 2003). Future ideas that could be developed from this experiment would be to apply it to the examination of different strains of *M. tuberculosis* and the identification of their *mutT*'s for resistance patterns

to see if there was a correlation between differences in this gene and the presence of heteroresistance. However recent evidence suggests that *mutT* polymorphisms do not increase the prevalence of drug resistance (Lari et al. 2006). Further work could involve the use of molecular probes to distinguish the resistant and sensitive populations of bacteria within the one isolate to demonstrate genotypic heteroresistance.

### 3.5 Conclusion

Phenotypic heteroresistance was demonstrated in this experiment to all isolates that were studied. Investigation of the *gyrA* of resistant clonal populations showed a wildtype sequence so no genetic mechanism for resistance was determined. Therefore it is hypothesised that this heteroresistance is an expression phenomenon which results in a change in an organism's phenotype. Standard culture antibiotic susceptibility testing obscures the detection of resistant sub-populations. The clinical relevance of this remains unclear.



## Chapter 4: An insight into the role of oxidative stress in *M. tuberculosis* evolution

### 4.1 Introduction

#### 4.1.1 Aim of this Chapter

The aim of this study is to review the literature describing resistance mutations in phenotypically resistant clinical isolates of *M. tuberculosis*. As rifampicin and pyrazinamide resistance usually occurs in the presence of a *katG* mutant, it is hoped that a review of rifampicin resistance mutations will provide an indication of the impact of oxidative stress to the *M. tuberculosis* genome. This may then provide an indication as to whether oxidative damage is the major driving force behind chromosomal mutations in drug resistance genes. If this hypothesis is correct, then damage to the guanine base will be observed by a high percentage of G → T mutations or on the opposite strand a C → A mutation. Alternatively it may result in the deamination of cytosine which are a source of C·G → T·A transitions.

## 4.2 Materials and method

Relevant studies were identified through computerised searches of the PubMed database (<http://www.ncbi.nih.gov/entrez/query.fcgi>). The search terms which were entered into the NCBI database were: rifampin AND tuberculosis AND sequencing AND resistance. The term rifampicin was also used and in subsequent searches, it was substituted with pyrazinamide. Single base pair changes conferring anti-microbial resistance that occurred on the sense strand of DNA were collated. The number of isolates, base pair change, amino acid affected and the amino acid change, isolates anti-microbial resistant as a result of a single base pair mutation were collected on an Excel (Microsoft) database. Countries of origin were collected as a control factor for geographic bias. Representatives of clinical isolates from outbreaks/clusters were counted once. Only single base pair mutations conferring resistance to rifampicin and pyrazinamide were counted. Deletions and inversions were excluded from the analysis. Double mutations were also excluded on the basis that it was unclear as to whether it was the double mutation or each mutation occurring singly that conferred resistance.

## 4.3 Results

### 4.3.1 Geographical Location of Isolates Analysed

Twenty four papers were analysed for mutations in *rpoB* (table 4.1) and 7 papers were analysed for mutations in *pncA* (table 4.2).

**Table 4.1:** The geographical location of *M. tuberculosis* isolates analysed for mutations in *rpoB*.

Country	No. of isolates	Reference
Australia	30	(Yuen, Leslie, & Coloe 1999)
Bangladesh	1	(Hirano, Abe, & Takahashi 1999)
Brazil	68	(Valim et al. 2000)
Canada	13	(Hirano, Abe, & Takahashi 1999)
Dubai	1	(Ahmad et al. 2000)
Germany	62	(Heep et al. 2001)
Greece	14	(Matsiota-Bernard, Vrioni, & Marinis 1998)
India	109	(Hirano, Abe, & Takahashi 1999; Mani et al. 2001; Siddiqi et al. 2002)
Indonesia	1	(Hirano, Abe, & Takahashi 1999)
Italy	33	(Pozzi et al. 1999)
Japan	103	(Taniguchi et al. 1996; Yang et al. 1998)
Latvia	13	(Tracevska et al. 2002)
Lebanon	11	(Ahmad et al. 2000)
Malaysia	14	(Hirano, Abe, & Takahashi 1999)
Mexico	28	(Ramaswamy et al. 2004)
Myanmar	14	(Hirano, Abe, & Takahashi 1999)
Nepal	2	(Hirano, Abe, & Takahashi 1999)
USA	66	(Cooksey et al. 1997; Nachamkin, Kang, & Weinstein 1997)
Peru	22	(Escalante et al. 1998)
Philippines	4	(Hirano, Abe, & Takahashi 1999)
Shanghai	32	(Fan et al. 2003)
South Africa	8	(Victor et al. 1999)
Spain	74	(Garcia, V et al. 2002; Garcia et al. 2001; Gonzalez et al. 1999)
Taiwan	40	(Hwang et al. 2003)
Thailand	13	(Hirano, Abe, & Takahashi 1999)
Turkey	43	(Brown, Tansel, & French 2000; Cavusoglu et al. 2002)
Yemen	21	(Hirano, Abe, & Takahashi 1999)
<b>TOTAL</b>	<b>840</b>	

**Table 4.2** The geographical location of *M. tuberculosis* isolates analysed for mutations in *pncA*.

Country	No. of isolates	Reference
Azerbaijan	2	(Mestdagh et al. 1999)
Bangladesh	3	(Mestdagh et al. 1999)
Canada	4	(Cheng et al. 2000;Mestdagh et al. 1999)
Japan	2	(Endoh et al 2002)
Korea	84	(Cheng et al. 2000;Lee, Lee, & Jung 2001)
Peru	8	(Escalante et al. 1998)
Scotland	3	(Mestdagh et al. 1999)
Singapore	5	(Lee et al. 2002)
Turkey	3	(Brown, Tansel, & French 2000)
<b>TOTAL</b>	<b>114</b>	

### 4.3.2 Exclusions from Study

A total of 7 papers analysed for mutations in *rpoB* were excluded from the study as they contained only amino acid data and it was not possible to deduce the mutational events which led to the amino acid substitution (Ahmad, Mokaddas, & Fares 2002;Hannan et al. 2001;Harris, Jr. et al. 2000;Kim et al. 2001;Mokrousov et al. 2003;Scorpio et al. 1997;Watterson et al. 1998). In some cases it was possible to deduce the base pair change which had occurred just from the amino acid information. Another paper was excluded as it reported induced spontaneous mutations in the lab strain *M. tuberculosis* H37Rv which is not a clinical isolate (Morlock, Plikaytis, & Crawford 2000). One study was eliminated due to errors in the reported mutations (El Baghdadi et al. 2003).

### 4.3.3 Mutation Detection

Most papers examined for this review sequenced a segment of *rpoB* containing the 81bp RRDR to identify any mutations (Heep et al. 2001; Pozzi et al. 1999; Siddiqi et al. 2002) or used a commercially available rapid test, the PCR based reverse hybridisation line probe assay (Inno-LiPA Rif TB test) (Brown, Tansel, & French 2000; Hirano, Abe, & Takahashi 1999; Tracevska et al. 2002) and real-time PCR using probes (Garcia, V et al. 2002; Torres et al. 2003). IS6110 typing was used to differentiate between isolates and identify clusters (Ramaswamy et al. 2004). Garcia et al evaluated PCR-ELISA to detect mutations in *M. tuberculosis* cultures and smear-positive specimens which agreed overall with DNA sequencing (Garcia et al. 2001). Direct sequencing was used to analyse the total length (561bp) of the *pncA* gene in all papers analysed (Lee et al. 2002; Lee, Lee, & Jung 2001; Mestdagh et al. 1999).

### 4.3.4 *rpoB* Mutations

The base pair change of C → T at codon Ser531Leu was the most reported single base pair mutation in the *rpoB* of clinical isolates of *M. tuberculosis* occurring in 456 out of 840 isolates (Table 4.3). Possibly even more significant, this mutation occurs in 54% of isolates including deletions, insertions, single and double base pair changes when looking at 1050 clinical isolates. The next most common mutation in *rpoB* is C → G (11%). C → A mutations occurred in 8 out of 840 isolates (0.9%). G → T mutations occurred in 21 out of 840 isolates (2.5%). The majority mutations reported occurred within the 81bp RRDR which is between codons 507 and 533 (Figure 4.1). Mutations occurring outside the RRDR were reported at codons 146 (Heep et al.

2001), 381 (Taniguchi et al. 1996), 490 (Cavusoglu et al. 2002) and 572 (Yuen, Leslie, & Coloe 1999). The mutation at codon Val146Phe is associated with high level resistance to rifampicin has also been demonstrated in *H. pylori* conferring resistance to rifampicin and results in a G → T change (Heep et al. 2000).

#### 4.3.5 *pncA* Mutations

Mutations were evenly distributed along *pncA* conferring resistance to pyrazinamide. There was no hotspot of mutation. Mutations in *pncA* were non-conserved, occurring at a wide number of codons (figure 4.2). Most frequent mutation in this gene is A → C occurring in 24 out of 114 isolates (21%). C → A and G → A mutations occurred equally in 5 out of 120 isolates (4.4%) (Table 4.3).

Other genes which were considered for use as a comparison to *rpoB* were *katG*, *inhA*, *rpsL* and *gyrA*. However there was insufficient data for analysis. Mutations in *katG* do not always confer resistance to isoniazid.

**Table 4.3:** The distributions of mutations within the *rpoB* gene of 840 clinical isolates of *M. tuberculosis*. The (\*) indicates transitions while all other nucleotide changes are transversions. The nucleotide changes which were relevant to the hypothesis are highlighted in grey.

Nucleotide Change	Codon (no. of isolates)	Total no. of isolates (%)
C→T *	381 (1), 521 (1), 522 (9), 526 (89), 531 (456)	556 (66.2)
C→G	511 (1), 522 (2), 526 (58), 531 (34)	95 (11.3)
C→A	509 (2), 513 (3), 522 (2), 526 (1)	8 (0.9)
G→C	528 (2)	2 (0.2)
G→T	146 (1), 490 (1), 516 (18), 527 (1)	21 (2.5)
G→A *	521 (1), 532 (2)	3 (0.3)
T→C *	511 (5), 533 (20)	25 (3)
T→A	0	0 (0)
T→G	0	0 (0)
A→C	513 (7), 518 (3), 526 (1)	11 (1.3)
A→G *	513 (1), 515 (1), 516 (4), 526 (24)	30 (3.6)
A→T	513 (6), 516 (69), 526 (13), 572 (1)	89 (10.6)

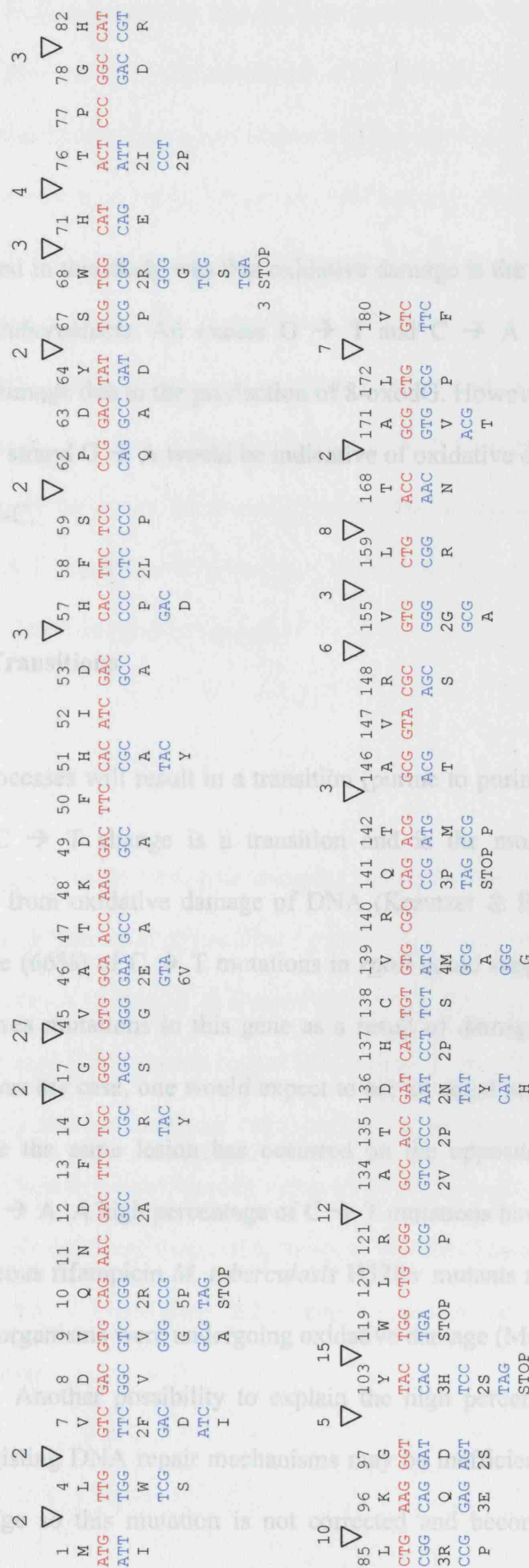




**Table 4.4:** The distributions of mutations within the *pncA* gene of 114 clinical isolates of *M. tuberculosis*. The (\*) indicates transitions while all other nucleotide changes are transversions. The nucleotide changes which were relevant to the hypothesis are highlighted in grey.

Nucleotide Change	Codon (no. of isolates)	Total no. of isolates (%)
<b>C→T *</b>	<b>10 (1), 46 (6), 51 (1), 76 (2), 134 (2), 141 (1), 142 (1), 171 (1)</b>	<b>15 (13.2)</b>
C→G	57 (1), 103 (1)	2 (1.8)
<b>C→A</b>	<b>46 (2), 62 (1), 148 (1), 168 (1)</b>	<b>5 (4.4)</b>
G→C	9 (1), 68 (1), 121 (1), 136 (1), 138 (1)	5 (4.4)
<b>G→T</b>	<b>1 (1), 7 (2), 136 (1), 180 (1)</b>	<b>5 (4.4)</b>
<b>G→A *</b>	<b>7 (1), 14 (1), 17 (1), 68 (3), 78 (1), 97 (3), 119 (1), 136 (2), 139 (4), 146 (1), 171 (1)</b>	<b>19 (16.7)</b>
T→C *	4 (1), 9 (1), 14 (1), 58 (2), 59 (1), 67 (1), 68 (2), 85 (1), 103 (3), 139 (1), 155 (1), 172 (1)	16 (14.0)
T→A	7 (1)	1 (0.9)
T→G	4 (1), 9 (1), 45 (1), 64 (1), 68 (1), 71 (1), 85 (3), 139 (1), 155 (2), 159 (1)	13 (11.4)
A→C	10 (5), 12 (2), 49 (1), 53 (1), 57 (1), 63 (1), 76 (2), 96 (1), 103 (2), 135 (2), 137 (2), 141 (3), 142 (1)	24 (21.1)
A→G *	8 (1), 10 (2), 47 (1), 51 (1), 82 (1), 96 (3)	9 (7.9)
A→T	0	0 (0)

**Figure 4.3:** The distribution of mutations within the *pncA* gene (accession no. Rv2043c) in *M. tuberculosis*. Numbers alongside altered amino acids indicate mutation frequencies, no number beside the amino acid were reported once. The arrowheads and numbers indicate the number of codons which were omitted from the diagram as they had no mutation.



## 4.4 Discussion

### 4.4.1 Hypothesis

The hypothesis tested in this study was that oxidative damage is the major mutational mechanism in *M. tuberculosis*. An excess  $G \rightarrow T$  and  $C \rightarrow A$  mutations would indicate oxidative damage due to the production of 8-oxodG. However, excess  $C \rightarrow T$  and on the opposite strand  $G \rightarrow A$  would be indicative of oxidative damage due to the production of 5-OH-C.

### 4.4.2 G·C $\rightarrow$ A·T Transitions

Most mutagenic processes will result in a transition (purine to purine / pyrimidine to pyrimidine). The  $C \rightarrow T$  change is a transition and is the most common base substitution arising from oxidative damage of DNA (Kreutzer & Essigmann 1998). The high percentage (66%) of  $C \rightarrow T$  mutations in *rpoB* could support the idea that oxidative stress drives mutations in this gene as a result of damage to dC residues (table 4.3). If this was the case, one would expect to see an equal percentage of  $G \rightarrow A$  transitions where the same lesion has occurred on the opposite strand. In this review, 0.3% are  $G \rightarrow A$ . A high percentage of  $C \rightarrow T$  mutations have been observed *in vitro*, in spontaneous rifampicin *M. tuberculosis* H37Rv mutants and there was no evidence that these organisms were undergoing oxidative damage (Morlock, Plikaytis, & Crawford 2000). Another possibility to explain the high percentage of  $C \rightarrow T$  mutations is that existing DNA repair mechanisms may be inefficient in recognising this base pair change so this mutation is not corrected and becomes fixed in the

population. However this possibility can be eliminated when looking at *pncA*. If oxidative damage, as a result of the deamination of cytosine, is the major driving force behind mutations in *M. tuberculosis*, then a high percentage of C → T mutations would have been observed in *pncA* which was not the case (table 4.4). 13.2% of mutations were C → T and on the opposite strand 16.7% were G → A. The deamination of cytosine can produce uracil which can cause a G·C → A·T transition unless the uracil is removed by a uracil DNA glycosylase encoded by the *ung* gene in *M. tuberculosis* (Handa, Acharya, & Varshney 2001). The high percentage of C → T transitions in *rpoB* may be due to the methylation of guanine which is a well known inducer of G·C → A·T transitions (Hoffmann, Crowley, & Theophiles 2002) which was observed in this study in *rpoB* but not *pncA*.

#### 4.4.3 Transversions

The next most common single base pair mutation in *rpoB* after C → T is C → G which is a transversion (purine to pyrimidine / pyrimidine to purine) and occurs less frequently than transitions (Schaaper & Dunn 1991), probably because it results in a greater structural change. Transversions occur less frequently than transitions in *rpoB* (table 4.3). It is likely that transversions cause lesions which are easily identified by DNA repair mechanisms and subsequently repaired more efficiently. It is not likely that the damage to dG residues is occurring as there is a low percentage of G·C → T·A observed in *rpoB* (3.4%) and *pncA* (8.8%) which suggests that oxidative damage is not driving mutations.

The low frequency of  $G \rightarrow T$  and  $C \rightarrow A$  mutations in *rpoB* may indicate that *M. tuberculosis* is sufficiently competent in repairing oxidised guanine induced by reactive oxygen intermediates. This does not seem surprising as this obligate pathogen has evolved to survive within the macrophage. The MutM/MutY proteins mediate this repair.

There was a low occurrence of  $A \cdot T \rightarrow C \cdot G$  (1.3%) transversions in *rpoB*. These transversions would have arisen from the misincorporation of adenine opposite 8-oxodGTP.

The isolates in many of the studies were isoniazid resistant so have a reduced capacity to deal with oxidative stress. This would result in a higher than predicted proportion of  $C \cdot G \rightarrow T \cdot A$  and  $G \cdot C \rightarrow T \cdot A$ , but this was not observed.

#### 4.4.4 *pncA* Mutations

Single nucleotide mutations are the major form of genetic change in *pncA*. Lee et al reported 69 out of 92 pyrazinamide resistant isolates had single nucleotide mutations (Lee, Lee, & Jung 2001). They found that mutations in this gene were too diverse to assign any geographic preference. Single mutations in *pncA* encoding pyrazinamide resistance are considered the major resistance mechanism in *M. tuberculosis* making it useful as a control gene in this comparative analysis to *rpoB*. There is a higher tendency towards nucleotide deletions and insertions in *pncA* compared to *rpoB*. Endogh et al noted that approximately 40% of amino acid substitutions involved a replacement with a proline residue (Endoh et al. 2002).

#### 4.4.5 Limitations of the Study

The analysis of *pncA* only incorporated 7 research articles so the scope of the information was limited. This is because the *pncA* is not frequently sequenced for mutation analysis. Also there is no hotspot of mutation so the whole gene must be analysed. The possibility that the whole *pncA* gene might be considered a hotspot of mutation within the *M. tuberculosis* genome cannot be ruled out. There are several other limitations that must be considered for this study. A highly diverse mutation profile has been reported in *pncA* and this observation in pyrazinamide resistant isolates is unique among all drug resistance genes in *M. tuberculosis*. There are 1235 possible single base pair mutations in *pncA* that can result in an amino-acid change or a stop codon (Brown, Tansel, & French 2000) of which at present 87 mutations have been reported. In each paper analysed, new and diverse *pncA* mutations were found. Such diversity makes it not an ideal control gene for comparison to *rpoB*. However this could not be avoided as sequence analysis on *katG*, *inhA*, *gyrA*, *gyrB* and *rpsL* genes was limited as mutations in these genes do not always confer resistant phenotypes so are not frequently analysed.

Other limitations in this study are that only mutations which confer a resistant phenotype were analysed so mutations which do not cause the development of resistance were not identified. Single nucleotide mutations which may have been caused by oxidative damage which do not lead to a detectable change in the phenotype are missed. Also mutations in genes other than those associated with drug resistance are missed. In the case of *rpoB*, only mutations which retain enzyme

function are examined. Segments of *rpoB* were analysed which incorporated the 81bp RRDR are frequently performed. However none of the papers sequenced the whole *rpoB* gene because of its length (3519bp), so already a bias is introduced, as there is an assumption that any mutations will be captured within the RRDR. The whole *pncA* gene was analysed for mutations. Only single base pair mutations could be counted to eliminate the likelihood that other mutations were caused by oxidative stress. Also, in studies clusters and outbreaks may be misrepresented, this was eliminated when possible by counting the number of isolates once. In most studies clinical isolates were isoniazid resistant so would have had a reduced capacity to deal with oxidative stress thereby, more mutations could be attributed to this functioning deficit. In some cases the presence of silent mutations may have been overlooked therefore not giving a real idea as to what mutations were occurring in the gene. This shows the importance in determining nucleotide base changes in order to predict amino acid sequence and hence antibiotic susceptibility. The only silent mutations in this review were found in *rpoB* at amino acid Leu-521 (Siddiqi et al. 2002; Yang et al. 1998). All the mutations in this study were observed in clinical isolates. However the pattern observed *in vivo* resembles the pattern of mutation observed *in vitro* (Morlock, Plikaytis, & Crawford 2000).

#### 4.4.6 Fitness Determines the Distribution of *rpoB* Mutations

The C → T mutation occurring at the Ser531Leu position was most common (Billington, McHugh, & Gillespie 1999), with 53% of mutations in *rpoB* of this type. It is notable that other C → T and G → A mutations were rare (12.53%). Billington et al selected for spontaneous mutations to rifampicin *in vitro* in *M. tuberculosis* H37Rv

and the 3 resistance patterns were detected by PCR-SSCP (single strand conformation polymorphism) analysis. They detected the Ser531Leu, His526Tyr and the His526Arg mutations. These mutations were then correlated with the frequency of clinical isolation in Musser's review (Musser 1995). The Ser531Leu mutation had the greatest relative fitness even in one isolate it had a greater relative fitness than the susceptible parent. Strains with the Ser531Leu mutation are better able to survive and are seen more frequently in rifampicin resistant clinical isolates. Mariam et al have recently confirmed these results (Mariam et al. 2004). They also measured the growth rates in a macrophage cell line with a virulent clinical isolate of *M. tuberculosis*. However they did not look at the point mutation resulting in the change Ser531Leu but they found that the mutation occurring at the same site Ser531Trp, associated with high-level rifampicin resistance, had the lowest fitness of the mutants analysed and interestingly is rarely isolated from patients. In this review there were 34 mutations of this type out of 853 isolates (4%). The distribution of rifampicin resistance is likely to be determined by fitness rather than exposure to mutagenic oxygen intermediates. However it is worth bearing in mind that fitness estimates are dependent on assay conditions *in vitro* and correlating these results with frequencies determined clinically should be interpreted with care.

Some resistance mutations appear to incur no fitness cost when measured *in vitro* for example *katG* mutations in isoniazid resistant *M. tuberculosis* (Pym, Saint-Joanis, & Cole 2002) so this argument may not be generalised. The fitness deficit resulting from resistance mutations in *rpoB* may be a feature of this gene. Similar findings have been reported in *rpoB* in *S. aureus* mutants which had reduced fitness (Wichelhaus et al.



2002). Thus we conclude that the distribution of mutations causing rifampicin resistance is likely to be determined by fitness of mutant strains.

#### 4.4.7 Absence of Mismatch Repair

Mizrahi and Anderson performed an *in silico* analysis of the DNA repair genes within the *M. tuberculosis* genome and failed to find any homologues of the *mutH*, *mutL*, *mutS*, *dam*, *dcm* and *vcr* genes which are all involved in mismatch repair (Mizrahi & Andersen 1998). The lack of a mismatch repair system has been demonstrated experimentally in *M. smegmatis* (Springer et al. 2004). Strains defective in these genes preferentially stimulate transitions and some frameshifts (Choy & Fowler 1985; Cox 1976; Glickman 1979). This may explain the high number of C → T transitions that are observed in the RRDR of *rpoB*. However the frequency of spontaneous mutations in *in vitro* cultures of *M. tuberculosis* is similar to that found in other bacteria carrying the mismatch repair systems which suggest other DNA repair mechanisms must be present (David & Newman 1971).

Schaaper & Dunn analysed spontaneous mutation in the *E. coli lacI* gene and of 414 independent mutations 70.8% were base substitutions and of these 47% were G·C → A·T transitions (Schaaper & Dunn 1991). They found that the occurrence of A·T → G·C and G·C → A·T transitions were reduced by mismatch repair. Miller & Low suggested that G·C → T·A and A·T → T·A transversions are a consequence of SOS processing of spontaneous lesions (Miller & Low 1984). The SOS response facilitates the generation of mutations when the bacterial cell is undergoing adverse conditions in order to provide a last gasp chance for the cell to survive. It is likely that mutations

of this type did occur within *rpoB*. The G·C → A·T transitions observed in this study in *rpoB* are arising from sources which are not subject to correction (Mizrahi & Andersen 1998).

#### 4.5 Conclusion

In this chapter, the hypothesis that oxidative damage is the major driving force behind mutations in *M. tuberculosis* is tested. There is evidence in the *rpoB* gene that oxidative damage leads to C → T base substitutions (induced by 5-OH-C) but this is not mirrored in the *pncA* gene. Also the opposite mutation G → A occurs at < 0.4% in *rpoB*. Our analysis shows that G·C → T·A mutations occur at low frequencies in both genes (4.4%) which may indicate that *M. tuberculosis* is sufficiently competent in the repair of oxidised guanine mediated by the MutM/MutY proteins. This leads us to conclude that oxidative damage is not the driving force behind mutations in the *M. tuberculosis* genome and instead it is the relative fitness of the mutant strain coupled with the resistance phenotype that permits it to survive and be detected.

## Chapter 5: Protection against oxidative damage

### 5.1 Introduction

#### 5.1.1 Aim of Chapter

It is proposed that isoniazid resistance through mutation in *katG* leads to an elevated spontaneous mutation rate due to a reduction in an organism's capacity to deal with oxidative stress. This could ultimately lead to an increased acquisition of drug resistance and the development of MDR-TB. It has been assumed that multi-drug resistance develops because of the accidental exposure of patients to single agents due to resistance, poor penetrating ability of the anti-tubercular compounds into the body compartments where the bacteria reside (figure 1.6) or due to random spontaneous mutation (section 1.12.3). It could be hypothesised that resistance to isoniazid develops before rifampicin, due to the generation of a mutator phenotype as a result of mutation in *katG*. Antagonism between mutations conferring resistance to streptomycin in *rpsL* and to rifampicin in *rpoB* has been confirmed in *M. smegmatis* (Karunakaran & Davies 2000) and in *E. coli* (Chakrabarti & Gorini 1975). So it is possible that there is antagonism between rifampicin and isoniazid resistance generating mutations. To investigate the hypothesis that a strain with a mutation in *katG* is hypermutable, the rate and nature of spontaneous mutants and hydrogen peroxide induced mutants to rifampicin of a panel of isoniazid resistant *M. tuberculosis* strains were analysed. These resistant strains all have different *katG* alleles with varying levels of catalase activity and were compared to the wild-type H37Rv. The results would demonstrate whether there is an increased acquisition of resistance due to the reduction in the organism's capacity to deal with oxidative stress induced by adding hydrogen peroxide.

## 5.2 Materials and Method

### 5.2.1 Strain Characteristics

A panel of isogenic strains with different *katG* genotypes obtained from the Pasteur Institute were investigated in this experiment (table 5.1). They were assembled by transforming mutant *katG* genes (on pKINT, a mycobacterial integration vector) into INH34, a  $\Delta furA$ -*katG* clinical isolate. All strains had different levels of catalase-peroxidase activity (Pym, Saint-Joanis, & Cole 2002). These strains were originally generated for the investigation of the effect of mutations in the catalase gene on the bacterial fitness of *M. tuberculosis* (figure 5.1). The strains were donated by Prof. Stewart Cole.

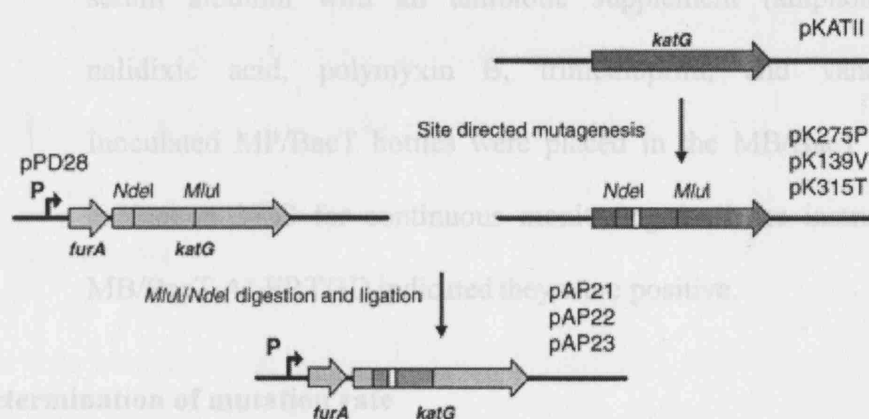
**Table 5.1: Strain genotype and corresponding enzymatic activity (Data from Pym et al 2002)**

Strain	Relevant feature	Catalase Activity <sup>a</sup>	Peroxidase Activity <sup>a</sup>
INH34 <sup>pPD28</sup>	pKINT with 4.8kb fragment spanning the <i>furA</i> - <i>katG</i> locus of <i>M. tuberculosis</i> .	1.87 ± 0.26	0.054 ± 0.002
INH34 <sup>pAP01</sup>	pPD28 with deletional interruption of <i>katG</i>	ND <sup>b</sup>	ND
INH34 <sup>pAP21</sup>	pPD28 with Thr275Pro mutation in <i>katG</i> .	ND	ND
INH34 <sup>pAP22</sup>	pPD28 with Ala139Val mutation in <i>katG</i> .	1.96 ± 0.44	0.043 ± 0.003
INH34 <sup>pAP23</sup>	pPD28 with Ser315Thr mutation in <i>katG</i> .	1.66 ± 0.18	0.046 ± 0.001
H37Rv	Fully functional <i>katG</i>	3.81 ± 0.17	0.11 ± 0.005

<sup>a</sup> Mean activity in whole-cell protein extracts in units per minute per milligram ± standard deviation from three experiments.

<sup>b</sup> ND, not detected.

**Figure 5.1:** Construction of plasmids to complement the  $\Delta furA$ -*katG* strain of *M. tuberculosis* with different *katG* alleles to generate pAP21, pAP22 and pAP23. Diagram from Pym et al 2002. The *E. coli* expression vector pKATII carried *katG* of *M. tuberculosis*. Site directed mutagenesis of *katG* was carried out by transferring a *NdeI*-*MluI* fragment spanning the various point mutations to the equivalent sites in pPD28 to create pAP21,22 and 23.



P: *katG* promoter

## 5.2.2 Determining the requirement of the strains for catalase

1. Strains INH34<sup>pAP01</sup>, INH34<sup>pAP23</sup> and INH34<sup>pPD28</sup> were cultured in 10mL 7H9 broth – ADC broth.
2. The broths were harvested by centrifugation at 3,000 g for 30 min and the supernatant was discarded. This washing process was repeated.
3. The pellets were re-suspended with 4mL 7H9 broth – 10% albumin dextrose complex (AD) broth.
4. Broths were incubated at 37°C for 2 weeks.

5. To check the viability of the cultures;

- a. An aliquot of each isolate was plated onto 7H10 agar. Plates were incubated in plastic bags at 37°C for up to 8 weeks.
- b. Neat and 1/1000 dilutions of INH34<sup>pAP01</sup>, INH34<sup>pAP23</sup> and INH34<sup>pPD28</sup> were prepared in 7H9 broth. Each dilution (0.5mL) was inoculated into an MP/BacT bottle (bioMérieux, Durham, N.C.) containing 10 mL of modified Middlebrook 7H9 broth with casein, catalase, and bovine serum albumin with an antibiotic supplement (amphotericin B, nalidixic acid, polymyxin B, trimethoprim, and vancomycin). Inoculated MP/BacT bottles were placed in the MB/BacT incubator cabinet at 37°C for continuous monitoring until the instrument the MB/BacT ALERT/3D indicated they were positive.

### 5.2.3 Determination of mutation rate

The mutation rate was determined by the *po* method as described (section 2.5). The mutation rate and mutation frequency of strains INH34<sup>pAP01</sup>, INH34<sup>pAP23</sup> and INH34<sup>pPD28</sup> were determined after exposure to hydrogen peroxide (section 2.5.1 and 2.6).

### 5.2.4 Screening of rifampicin resistant colonies for mutations within *rpoB*

Resistant colonies were picked from 7H10 agar plates and the procedure was followed as described in section 2.8.2.

## 5.3 Results

### 5.3.1 Pure growth of strains on media

All strains were grown on 7H10 agar.

### 5.3.2 Growth of strains without catalase

Using method 5.2.2. INH34<sup>pAP01</sup>, INH34<sup>pAP23</sup> and INH34<sup>pPD28</sup> did not grow in the absence of catalase either on solid or liquid media so subsequent mutation rate experiments had to be performed in the presence of catalase.

### 5.3.3 Mutation rates

Mutation rates were determined using method 2.5 (table 5.2). The rows shaded in grey in tables 5.2 and 5.3 indicate treatment with 8mM H<sub>2</sub>O<sub>2</sub> for 24hr, using method 2.5.1. Results are summarised in table 5.3.

**Table 5.2:** Mutation rate for each strain

Strains	Mean cfu/ml	No. of cultures with no mutants ( $p_0$ )	No. of mutations per culture ( $m$ )	Mutation rate ( $\mu$ )
INH34 <sup>pAP01</sup>	$7.89 \times 10^6$	0.28	1.27	$1.6 \times 10^{-7}$
INH34 <sup>pAP01</sup>	$2.11 \times 10^6$	0.5	0.69	$3.27 \times 10^{-7}$
INH34 <sup>pAP01</sup>	$2.42 \times 10^6$	0.32	1.14	$4.71 \times 10^{-7}$
INH34 <sup>pAP01</sup>	$3.78 \times 10^6$	0.2	1.61	$4.26 \times 10^{-7}$
INH34 <sup>pAP01</sup>	$6 \times 10^5$	0.8	0.22	$3.67 \times 10^{-7}$
INH34 <sup>pAP21</sup>	$1.13 \times 10^7$	0.31	1.17	$1.03 \times 10^{-7}$
INH34 <sup>pAP21</sup>	$4.75 \times 10^7$	0.12	2.12	$4.46 \times 10^{-7}$
INH34 <sup>pAP22</sup>	$5.5 \times 10^6$	0.75	0.29	$5.27 \times 10^{-8}$
INH34 <sup>pAP22</sup>	$1.33 \times 10^5$	0.42	0.87	$6.54 \times 10^{-6}$
INH34 <sup>pAP23</sup>	$6.64 \times 10^5$	0.12	2.12	$3.2 \times 10^{-6}$
INH34 <sup>pAP23</sup>	$2.28 \times 10^6$	0.4	0.92	$4.04 \times 10^{-7}$
INH34 <sup>pAP23</sup>	$6.42 \times 10^7$	0.52	0.65	$1.01 \times 10^{-8}$
INH34 <sup>pAP23</sup>	$6.75 \times 10^6$	0.4	0.92	$1.36 \times 10^{-7}$
INH34 <sup>pAP23</sup>	$1.16 \times 10^6$	0.29	1.24	$1.07 \times 10^{-6}$
INH34 <sup>pPD28</sup>	$1.45 \times 10^6$	0.28	1.27	$8.76 \times 10^{-7}$
INH34 <sup>pPD28</sup>	$7.9 \times 10^6$	0.25	1.39	$1.76 \times 10^{-6}$
INH34 <sup>pPD28</sup>	$7.2 \times 10^5$	0.2	1.61	$2.24 \times 10^{-6}$
INH34 <sup>pPD28</sup>	$6.42 \times 10^5$	0.4	0.92	$1.43 \times 10^{-6}$
H37Rv	$5.3 \times 10^6$	0.5	0.69	$1.3 \times 10^{-7}$
H37Rv	$2.4 \times 10^6$	0.56	0.58	$2.4 \times 10^{-7}$

■ 8mM H<sub>2</sub>O<sub>2</sub> for 24hr



**Table 5.3:** Summary of strains and mutation rates

Strain Designation	Number of cultures studied	Median Mutation Rate	Range
INH34 <sup>pAP01</sup>	3	$3.2 \times 10^{-7}$	$1.6 - 4.71 \times 10^{-7}$
INH34 <sup>pAP01</sup>	2	$4 \times 10^{-7}$	$3.67 - 4.26 \times 10^{-7}$
INH34 <sup>pPD28</sup>	4	$2 \times 10^{-6}$	$8.76 \times 10^{-7} - 2.24 \times 10^{-6}$
INH34 <sup>pAP21</sup>	2	$2.7 \times 10^{-7}$	$1.03 - 4.46 \times 10^{-7}$
INH34 <sup>pAP22</sup>	2	$3.3 \times 10^{-6}$	$5.27 \times 10^{-8} - 6.54 \times 10^{-6}$
INH34 <sup>pAP23</sup>	3	$4.04 \times 10^{-7}$	$1.01 \times 10^{-8} - 3.2 \times 10^{-6}$
INH34 <sup>pAP23</sup>	2	$6.03 \times 10^{-7}$	$1.36 \times 10^{-7} - 1.07 \times 10^{-6}$
H37Rv	1	$1.3 \times 10^{-7}$	----
H37Rv	1	$2.4 \times 10^{-7}$	-----

■ 8mM H<sub>2</sub>O<sub>2</sub> for 24hr

#### 5.3.4 Mutation frequency of INH34<sup>pAP01</sup>, INH34<sup>pAP23</sup> and INH34<sup>pPD28</sup>

The method was followed as described in section 2.6. There was no growth from the cultures in the absence of catalase. However there was growth before the catalase had been removed on the plates (table 5.4).

**Table 5.4:** Colony counts from 3 replicate experiments before the catalase had been removed from the media.

Strain	Mean Cfu/mL
INH34 <sup>pAP01</sup>	$2.38 \times 10^5$
INH34 <sup>pAP23</sup>	$5 \times 10^2$
INH34 <sup>pPD28</sup>	$1.1 \times 10^2$

### 5.3.5 Sequencing of *rpoB*

The region of the *rpoB* incorporating codon 146 of 35 isolates (primer set 1) was sequenced and there were no mutations. Mutations were found at this site in previous studies of rifampicin resistant clinical isolates of *M. tuberculosis* (Heep et al. 2001; Jenkins et al. 2005). The RRDR of 66 rifampicin resistant mutants were sequenced (16 using primer set 2 and 50 using primer set 3) and mutations were found in 3 isolates; 2 with Ser531Leu and 1 with His526Asp (figure 5.2).

**Figure 5.2: Alignment of *rpoB* sequences showing the mutations detected. A C→G change at codon 526 and a C→T change at codon 531.**

```

rpoB  ACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTG
seq1  ACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTG
seq2  ACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTTGGCGCTG
seq3  ACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTTGGCGCTG
      *****

```

### 5.4.2 Estimating Mutation Rates using the $p_0$ Method

There are several methods for estimating mutation rates. For the purpose of these experiments the method used to determine the mutation rate is the  $p_0$  method (Luria & Delbrück 1943) which can be used if mutants are selected on liquid or solid media (section 1.13.2). This method was chosen as it is appropriate when low mutation rates are expected which is the case when mutants are selected on rifampicin. This method counts the number of cultures without mutants and should only be used when  $p_0$  is between 0.1 and 0.7. In this experiment,  $p_0$  was 0.12-0.80 (table 5.2). It is a simple and reliable method but it can produce highly variable results. For the Poisson distribution

## 5.4 Discussion:

### 5.4.1 Proposed Hypothesis

In this experiment, we investigate the mutation rate of isoniazid resistant *M. tuberculosis* strains to rifampicin. The strains used have deletions in the catalase gene impairing the bacteria's defence against oxygen free radicals encountered frequently inside the macrophage (Manca et al. 1999). The hypothesis that a strain with a *katG* mutation leads to a mutator phenotype is proposed. The effect of oxidative stress is mutagenic because the organism has a reduced capacity to deal with the reactive oxygen species. Resistance to rifampicin is investigated because it is often used as an indicator of MDR-TB due to the low rate of *M. tuberculosis* mutation to resistance against this anti-microbial, with an estimated resistance prevalence of 1 in  $10^8$  bacilli in drug-free environments (WHO 2004).

### 5.4.2 Estimating Mutation Rates using the $p_0$ Method

There are several methods for estimating mutation rates. For the purpose of these experiments the method used to determine the mutation rate is the  $p_0$  method (Luria & Delbrück 1943) which can be used if mutants are selected on liquid or solid media (section 1.13.2). This method was chosen as it is appropriate when low mutation rates are expected which is the case when mutants are selected on rifampicin. This method counts the number of cultures without mutants and should only be used when  $p_0$  is between 0.1 and 0.7. In this experiment,  $p_0$  was 0.12-0.80 (table 5.2). It is a simple and reliable method but it can produce highly variable results. For the Poisson distribution

method,  $m$  (number of mutations per culture) should be greater than 0.3 but less than 2.3. In this experiment,  $m$  was 0.22-2.12 (table 5.2). The second parameter for consideration is the number of parallel cultures,  $C$ , to be set up to represent the bacterial population sufficiently (section 1.14.4.2). For this experiment  $C$  was 25, where about 50% of the cultures contained mutants. The third parameter to consider is  $N_0$  which was 5,000 cells/mL. This has been used as the starting inoculum previously (Boshoff et al. 2003) and it was sufficient to represent the population without generating too many mutants.

#### 5.4.3 INH34 Transformed Strains with Differing *katG* Alleles

The strains used in this experiment were generated by Pym et al for investigation into the virulence of *katG* deficient genes in *M. tuberculosis* (Pym, Saint-Joanis, & Cole 2002) (table 5.1). The strain with the *katG* Thr275Pro mutant, INH34<sup>pAP21</sup>, had no catalase-peroxidase activity detected and a high level of resistance to isoniazid, resulted in a greatly reduced virulence in the mouse model. The strain INH34<sup>pAP23</sup>, which had the Ser315Thr mutation, did not result in loss of virulence in the mouse model yet retained a clinically significant level of isoniazid resistance. These mutations may therefore favour the emergence of MDR strains as they lead to minimal fitness reduction. This is the most frequently observed mutation *in vivo* because it confers isoniazid resistance yet still retains significant catalase activity. The early observation made by Middlebrook which describes attenuated isoniazid resistant strains in a guinea pig model is only correct for some resistant strains (Middlebrook 1954).

#### 5.4.4 Mutation Rate Results

The mutation rates obtained are in the range of  $5.27 \times 10^{-8}$  and  $6.54 \times 10^{-6}$  per cell generation (table 5.3). The INH34 strains were found to have similar mutation rates to H37Rv. There was no evidence that hydrogen peroxide treatment had any effect on the rate of mutation. We found the mutation rate of H37Rv, using the  $p_0$  method to be  $1.3 \times 10^{-7}$  per cell division. Werngren and Hoffner, using the Luria-Delbrück fluctuation test, calculated the mutation rate of H37Rv to be  $8.6 \times 10^{-9}$  and the mutation frequency of H37Rv to be  $8.6 \times 10^{-8}$  (Werngren & Hoffner 2003). The difference between the results was probably because of the different methods used. The mutation frequency only simply averages the fraction of mutant bacteria in a few replicate cultures and does not take into account that during exponential growth of mutants, low probability events occurring early will have huge consequences during the growth of a bacterial population.

The mutation rate of strains with no catalase-peroxidase activity INH34<sup>pAP01</sup> and INH34<sup>pAP21</sup> were within the same order of magnitude ( $10^{-7}$ ) as strains which had enzymatic activity; INH34<sup>pPD28</sup>, INH34<sup>pAP22</sup>, INH34<sup>pAP23</sup> and H37Rv (table 5.3). The rate of mutation was in the same order of magnitude when compared to strains with the addition of hydrogen peroxide. It was expected that the mutant strains reduced capacity for *katG* to metabolise H<sub>2</sub>O<sub>2</sub> would lead to a higher mutation rate when the bacteria would be stressed due to exposure to O<sub>2</sub> radicals. The addition of this oxidizing agent to the environment did not appear to make a significant impact on the bacteria which were already dealing with the H<sub>2</sub>O<sub>2</sub> which was being produced endogenously by the respiratory chain. The treatment of 8mM H<sub>2</sub>O<sub>2</sub> for 24hr was

sufficient to induce oxidative stress as it has been shown that 4mM H<sub>2</sub>O<sub>2</sub> for 24hr resulted in mutation induction in *M. tuberculosis* (Boshoff et al. 2003) so there should have been an increase in mutation rate. Having a fully functional *katG* made no impact on the rate of mutation.

#### 5.4.5 Presence of Exogenous Catalase

In order to determine the mutation rate in the presence of H<sub>2</sub>O<sub>2</sub> it was necessary to culture the strains without catalase. The addition of catalase to the media was essential, however, for the growth of the strains so this could not be avoided. It is unknown to what level of this exogenous source of enzyme would have helped the bacteria to break down hydrogen peroxide into harmless by-products. Other authors have investigated oxidative stress conditions have added H<sub>2</sub>O<sub>2</sub> to Middlebrook 7H9 broth with ADC enrichment (contains catalase) and did not take this factor into account (Dhandayuthapani et al. 1996;Hu et al. 2004;Hu et al. 1999;Hu & Coates 1999;Jensen-Cain & Quinn 2001;Kendall et al. 2004). All INH34 derived strains had an impaired growth rate in the presence of catalase compared with H37Rv; observed by longer incubation times. The removal of this enzyme resulted in a further reduction and even cessation of growth. This leads to the conclusion that the INH34 strains require exogenous catalase for growth and protection against natural oxides present during growth. This is not the case for the lab strain H37Rv which grows without the addition of catalase as it has adapted for *in vitro* growth (Boshoff et al. 2003;Manca et al. 1999).

In order to overcome the interaction of the hydrogen peroxide with catalase, the strains were grown with catalase, which was then removed before the addition of hydrogen peroxide to the medium. However, there was no growth on the 7H10 plates with or without rifampicin. The inoculum in this experiment was lower than when determining mutation rates so this may have been a factor (table 5.4). It is more likely that the removal of the catalase and subsequent addition of hydrogen peroxide was detrimental to the cultures.

#### 5.4.6 Compensation by *ahpC* for Deficiency of Catalase-Peroxidase Activity

Compensatory mutations could occur to counterbalance for a loss of bacterial fitness as a result of resistance conferring mutations (Lipsitch 2001). Mutations have been described in the *ahpC* promoter region which upregulate AhpC, an alkyl hydroperoxide reductase which may compensate for catalase-peroxidase deficiencies as a result of mutations in *katG* (Sherman et al. 1996; Springer et al. 2001) (section 1.11.2.2.d). AhpC belongs to the family of peroxiredoxins which are involved in antioxidant protection (Guimaraes et al. 2005). The clinical isolate INH34 has an upregulated promoter region of *ahpC* so any differences in the mutation rate could not be due to the emergence of this compensatory mutation (Pym et al. 2001). The levels of *ahpC* expression must have been sufficient to deal with the levels of oxidative stress in the media even when catalase activity is absent in INH34<sup>pAP01</sup> and INH34<sup>pAP21</sup>. This would explain why there was no difference in the mutation rate between any of the INH34 *M. tuberculosis* strains transformed with differing *katG* alleles and the wild-type H37Rv. Mutations may be occurring in other genes also to further regulate the level of stress from the reactive oxygen species present in the

media. Other genes may have the ability to compensate for loss of *katG* activity. Single nucleotide polymorphisms have been reported in *ndh* in isoniazid resistant *M. tuberculosis* (Lee, Teo, & Wong 2001). This gene encodes NADH dehydrogenase which balances the NADH/NAD<sup>+</sup> ratio by transferring electrons to quinones of the respiratory chain (Miesel et al. 1998).

#### 5.4.7 Pattern of Mutations in *rpoB*

A 1079bp segment of *rpoB* (total length 3519bp), which includes the 81bp hotspot region of mutation, was screened. Only 3 out of 66 (4.5%) rifampicin resistant isolates had mutations within the RRDR of *rpoB* (figure 5.2). These results do not reflect the pattern of mutations commonly seen in rifampicin resistant clinical isolates with approximately 90% having mutations in this 81bp hotspot. A clinical study also showed a different pattern of mutations in *rpoB* among outbreak isoniazid resistant strains in North London (Jenkins et al. 2005). They reported the mutation Val146Phe, outside the hotspot region of mutation which has previously been described in clinical isolates (Heep et al. 2001). No mutations at this codon were observed in this study. There may have been mutations in the other region of *rpoB* but no genetic determinants of resistance have been uncovered in other gene segments outside the 81bp hotspot and codons 146, 490 and 572 (section 4.3.4). The mutants were isolated at 2µg/mL rifampicin (twice the MIC for *M. tuberculosis* H37Rv). A relationship between susceptibility to rifampicin and the pattern of mutations in *rpoB* has been observed. The colonies sequenced here therefore may have had low MIC's. Ohno et al showed that rifampicin resistant strains with MIC's of <32µg/mL had a different



pattern of mutation with 12.5% occurring in the RRDR and all strains with a MIC of  $\geq 64\mu\text{g/mL}$  having mutations within the hotspot region (Ohno et al. 1996).

The contribution of drug efflux pumps to drug resistance has been reported in *M. tuberculosis*, in particular *efpA* (Doran et al. 1997) and a tap-like pump (Ainsa et al. 1998; Siddiqi et al. 2004). The tap-like putative multi-drug efflux pump has been shown to confer resistance to aminoglycosides and tetracycline in *M. fortuitum* (Ainsa et al. 1998). Changes in efflux pumps may render the mycobacterial cell wall resistant to the accumulation of rifampicin (Piddock, Williams, & Ricci 2000). Rifampicin resistant mutants with no *rpoB* mutation might arise with enhanced efflux, mediating rifampicin resistance. There is evidence that mutations within genes encoding efflux pump mediate drug resistance (Nikaido 1996; Paulsen, Brown, & Skurray 1996).

The mutants isolated in this experiment were phenotypically resistant but may not be genotypically resistant. The resistant colonies may have been as a result of a change in the expression of a regulatory system or perhaps as a result of a previously uncharacterised mutation in another gene.

## 5.5 Conclusion

The hypothesis that isoniazid resistance through a mutation in *katG* reduces the organism's capacity to deal with oxidative stress and as a result leads to a mutator phenotype is proposed. There was no difference in mutation rate however between isoniazid sensitive and resistant organisms. Addition of hydrogen peroxide made no phenotypic impact on the mutation rate. This protection may have occurred as a result of the upregulation of *ahpC* which compensates for the diminished or absent levels of catalase-peroxidase activity to balance the levels of reactive oxygen species which damage the DNA. Upon screening of rifampicin resistant colonies, the pattern was different to that observed in resistant clinical isolates. Another genetic determinant of resistance may be present or perhaps it is just an expression of an unknown gene.

## **Chapter 6: The effect of sub-inhibitory concentrations of ciprofloxacin on the DNA repair responses of *M. tuberculosis***

### **6.1 Introduction**

To extend the observation that sub-inhibitory concentrations of quinolone increase the mutation rate of *M. fortuitum* (section 1.11.4.3) (Gillespie et al. 2005), microarray studies were performed to monitor the changes in the SOS response (section 1.14.3.1) of *M. tuberculosis* in response to sub-inhibitory doses of ciprofloxacin; a fluoroquinolone. The results were confirmed by quantitative reverse transcriptase PCR (qRT-PCR) Gene expression studies have been used to demonstrate the effects of norfloxacin on *E. coli* and it was shown to induce the SOS response (Shaw et al. 2003).

#### **6.1.1 The Use of DNA Microarrays**

Use of DNA microarrays facilitates the interrogation of RNA preparations extracted from *in vitro* cultivated bacteria (Stewart et al. 2002). This technology has allowed transcriptional profiles of bacteria to be examined in response to various stresses. The use of genome microarrays to examine the induction of *M. tuberculosis* genes in response to isoniazid treatment has been reported (Waddell et al. 2004; Wilson et al. 1999) and arrays are increasingly been used to identify unknown anti-tubercular drug specific changes which may reflect the mode of action (Betts et al. 2003; Waddell et al. 2004). Genomic technologies generate signature expression profiles for different drugs in *M. tuberculosis* and give insight into the metabolic effects of the drugs (Betts

et al. 2003). RNA response profiles are proposed to serve as a fingerprint of the mode of action of any given drug as one could predict that genes which were induced could compensate for the inhibition of the target pathway or respond to the toxicity of the drug; for example the induction of *ahpC*, *efpA* and *kasA* genes encoding fatty acid synthesis upon exposure to isoniazid (Wilson et al. 1999). Further insight can be obtained by monitoring gene expression during metabolic slow-down over the course of adaptation to nutrient starvation when the bacilli are in a non-replicating state. The role of genes involved in maintaining this state have been studied (Betts et al. 2002) with the intention of identifying potential targets for anti-tubercular agents active against non-replicating bacilli. The physiological response of *M. tuberculosis* to stress has been dissected in a wide range of array studies; heat shock (Stewart et al. 2002), carbon starvation (Betts et al. 2002;Dahl et al. 2003), aerobic growth (Wernisch et al. 2003), hypoxia or nitric acid (Bacon et al. 2004;Voskuil et al. 2003), iron limitation (Rodriguez et al. 2002) and SDS stress (Manganelli et al. 2002). Host expression and bacterial gene expression *in vivo* have also been reported (Keller et al. 2004;McGarvey, Wagner, & Bermudez 2004;Nau et al. 2002;Nau et al. 2003;Schnappinger et al. 2003;Talaat et al. 2004;Xu et al. 2003). Boshoff et al applied whole genome expression profiling to many different anti-microbial agents and also to diverse environmental changes to identify clusters of corresponding genes across a range of treatments (Boshoff et al. 2004). The complex environment found in phagosomes can be mimicked by exposing *M. tuberculosis* to acidic conditions and then transcriptional profiling has been used to determine the genes involved in this response (Fisher, Plikaytis, & Shinnick 2002).

### 6.1.2 Aim

The aim of this chapter is to investigate the hypothesis that sub-inhibitory concentrations of quinolones can increase the mutation rate of *M. tuberculosis* by inducing the error-prone SOS response. This was accomplished using gene expression profiling to monitor changes in the response of *M. tuberculosis* in response to  $\frac{1}{2}$  and  $\frac{1}{4}$  MIC concentrations of ciprofloxacin. The expression of *dnaE2* was investigated using qRT-PCR to validate the microarray data and to further investigate the SOS response.

## 6.2 Materials and method

### 6.2.1 Quinolone treatment of culture

1. Twenty-one 50mL cultures of exponentially growing *M. tuberculosis* H37Rv (NCTC 7416) were prepared in 7H9 – ADC broth at a concentration of  $5.5 \times 10^6$  cfu/mL.
2. Six 50mL cultures were exposed to 0.25mg/L of ciprofloxacin ( $\frac{1}{2}$  MIC) and six 50mL cultures were exposed to 0.125mg/L of ciprofloxacin ( $\frac{1}{4}$  MIC) over a period of 12hr. Untreated culture controls were included at each time point in which the same volume of SDW (2.5mL) was added instead of ciprofloxacin (3 controls at  $t = 0$ hr, 4hr and 12hr). These broth cultures were mixed and incubated at 37°C.
3. Colony counts were performed on each treatment condition and the dilutions  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  were plated on 7H10 agar plates (section 2.3).
4. RNA was extracted (section 2.7.3), cleaned up (section 2.7.4) and quantified (section 2.8.4).
5. Two hybridisations were carried out for each RNA sample (table 6.1).

**Table 6.1:** Experiments performed

Time of RNA extraction (hr)	0	4	12	4	12
Drug Conc. (X MIC)	none	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{4}$
Biological replicates	3	2	1	3	1
Technical replicates of each sample	2	2	2	2	2

6. The microarray and qRT-PCR procedures are outlined in section 2.8.7 and 2.9.

### **6.2.2 Bioassay: to check the activity of the batch of ciprofloxacin**

Approximately 300mL Mueller Hinton agar was poured into a bioassay dish (Nunc, Roskilde, Denmark) and left to set. A lawn of 0.5 McFarland *E. coli* ATCC 25922 was created and left to dry. Holes were cut using a cutter and labelled with different antibiotic concentrations. The antibiotic was diluted to incorporate a range of drug concentrations 0.5 – 0.004µg/mL. Into each hole 0.2mL of each dilution was added. The bioassay dish was incubated overnight at 37°C. The zones of inhibition were measured.

### 6.3 Results

Table 6.2 is the list of genes which were identified as being DNA repair genes which were further studied. Table 6.3 compares the cfu/mL of the different treatments showing that there was no reduction in bacterial numbers.

Genes were found to be differentially expressed comparing the various treatment exposures to untreated controls (table 6.5). Differential gene expression showed overall that genes were mostly down-regulated with fewer genes being up-regulated. Using ANOVA there were no genes significantly different at each time point between  $\frac{1}{2}$  and  $\frac{1}{4}$  MIC treatment. Figure 6.1 and 6.2 show the overlap of 105 genes at 4hr and 60 genes at 12hr between both treatments. There was an up-regulation of 17 different genes and a down-regulation of 12 different genes involved in DNA repair from the various treatments (table 6.6a-d). Most effects were observed after 4hr. After  $\frac{1}{2}$  MIC ciprofloxacin for 4hr there was a down-regulation of 6 genes and an up-regulation of 3 genes. After 12hr of the same treatment there was a down-regulation of 3 genes and an up-regulation of 10 genes. After  $\frac{1}{4}$  MIC ciprofloxacin for 4hr there was a down-regulation of 8 genes and an up-regulation of 7 genes. After 12hr of the same treatment there was a down-regulation of 3 genes and an up-regulation of 3 genes.

To validate the microarray data the expression of *dnaE2* was normalized to *sigA* using qRT-PCR.



**Table 6.2:** Results of a database search for genes involved in DNA repair, mutagenesis and recombination in *M. tuberculosis* which were further studied in this experiment. One hundred and ten genes were identified from Mizrahi and Andersen (Mizrahi & Andersen 1998) and a search of the Entrez Genome, NCBI database of the COG (clusters of orthologous groups of proteins) classification DNA replication, recombination and repair.

Gene	Ref	Gene	Ref	Gene	Ref
<i>ada, alkA</i>	M & A	<i>recR</i>	M & A	Rv2817c	NCBI
<i>arcA</i>	NCBI	<i>rpoB</i>	NCBI	Rv2819c	NCBI
<i>dinG</i>	M & A	<i>rpsF</i>	NCBI	Rv2820c	NCBI
<i>dinP, dinB</i>	M & A	<i>ruvA</i>	M & A	Rv2821c	NCBI
<i>dinX</i>	NCBI	<i>ruvB</i>	M & A	Rv2884	NCBI
<i>dnaB</i>	NCBI	<i>ruvC</i>	M & A	Rv2896c	NCBI
<i>dnaE1</i>	NCBI	Rv0586	NCBI	Rv2943	NCBI
<i>dnaE2</i>	NCBI	Rv0741	NCBI	Rv2944	NCBI
<i>dut</i>	M & A	Rv0938	NCBI	Rv2978c	NCBI
<i>embB</i>	NCBI	Rv1035c	NCBI	Rv3040c	NCBI
<i>fpg, mutM</i>	M & A	Rv1036c	NCBI	Rv3185	NCBI
<i>gnd</i>	NCBI	Rv1055	NCBI	Rv3198c	NCBI
<i>gyrA</i>	M & A	Rv1227c	NCBI	Rv3201c	NCBI
<i>gyrB</i>	M & A	Rv1688	NCBI	Rv3202c	NCBI
<i>lexA</i>	M & A	Rv1956	NCBI	Rv3380c	NCBI
<i>ligA</i>	M & A	Rv1985c	NCBI	Rv3386	NCBI
<i>linB</i>	NCBI	Rv2013	NCBI	Rv3394c	NCBI
<i>mfd</i>	M & A	Rv2017	NCBI	Rv3428c	NCBI
<i>mpg</i>	M & A	Rv2090	NCBI	Rv3430c	NCBI
<i>mutT</i>	M & A	Rv2100	NCBI	Rv3672c	NCBI
<i>mutT1</i>	NCBI	Rv2105	NCBI	Rv3856c	NCBI
<i>mutT3</i>	NCBI	Rv2106	NCBI	Rv3908	NCBI
<i>mutY</i>	M & A	Rv2119	NCBI	<i>sigE</i>	NCBI
<i>nei</i>	M & A	Rv2167c	NCBI	<i>sigG</i>	NCBI
<i>nfo, end</i>	M & A	Rv2177c	NCBI	<i>sigH</i>	NCBI
<i>nth</i>	M & A	Rv2191	NCBI	<i>ssb</i>	M & A
<i>ogt</i>	M & A	Rv2278	NCBI	<i>tagA</i>	M & A
<i>polA</i>	M & A	Rv2362c	NCBI	<i>topA</i>	M & A
<i>polC</i>	M & A	Rv2464c	NCBI	<i>ung</i>	M & A
<i>radA</i>	M & A	Rv2479c	NCBI	<i>uvrA</i>	M & A
<i>recA</i>	M & A	Rv2512c	NCBI	<i>uvrB</i>	M & A
<i>recB</i>	M & A	Rv2578c	NCBI	<i>uvrC</i>	M & A
<i>recC</i>	M & A	Rv2609c	NCBI	<i>uvrD</i>	M & A
<i>recD</i>	M & A	Rv2648	NCBI	<i>xthA</i>	M & A
<i>recF</i>	M & A	Rv2719c	NCBI	<i>yshC</i>	M & A
<i>recG</i>	M & A	Rv2791c	NCBI		
<i>recN</i>	M & A	Rv2816c	NCBI		

**Table 6.3:** Colony counts of bacteria comparing the number of bacteria recovered from each treatment demonstrating no reduction in bacterial numbers.

Time (hr)	Treatment (MIC)	Cfu/mL
0	None	$5.5 \times 10^6$
4	0.5	$5.3 \times 10^6$
4	0.25	$1.2 \times 10^7$
12	0.5	$7.8 \times 10^6$
12	0.25	$7.5 \times 10^6$

**Table 6.4:** The inhibition of *E. coli* ATCC 25922 using different dilutions of ciprofloxacin. The quality control (QC) range is 0.004 -0.016µg/mL.

Drug Conc (µg/mL)	Zone of inhibition (mm)
0.5	9
0.25	7
0.125	6
0.06	4
0.03	3
0.015	2.5
0.008	1
0.004	None

**Table 6.5:** Number of genes with statistically significant differences analysed using ImaGene™ and Bluefuse™ software. Parametric test, variances not assumed equal (Welch t-test). *P*-value cut-off 0.05, multiple testing correction: Benjamini and Hochberg False Discovery Rate. This restriction tested 3924 genes. About 5.0% of the identified genes would be expected to pass the restriction by chance.

Treatment Time (MIC)	No. of genes with statistically significant differences			
	Up-regulated		Down-regulated	
	ImaGene™	BlueFuse™	ImaGene™	Bluefuse™
4hr (0.25) v 0hr	40	31	108	105
4hr (0.5) v 0hr	127	26	262	109
12hr (0.25) v 0hr	77	37	144	42
12hr (0.5) v 0hr	102	9	143	6
12hr (0.25) v 4hr (0.25)	7	17	16	8
12hr (0.5) v 4hr (0.5)	0	0	1	0
4hr (0.25) v 4hr (0.5)	0	0	0	0
12hr (0.25) v 12hr (0.5)	0	0	0	0

**Table 6.6.a-d:** Down- and up- regulated genes from DNA repair, recombination and mutagenesis gene list (110 genes) with statistically significant differences among Time, MIC for 4hr and 12hr treatments with  $\frac{1}{2}$  and  $\frac{1}{4}$  MIC ciprofloxacin. Genes are ranked by ascending levels of expression as compared to untreated control at time 0hr. Parametric test, variances not assumed equal (Welch t-test). *P*-value cutoff 0.05, multiple testing correction: Benjamini and Hochberg False Discovery Rate. About 5.0% of the identified genes would be expected to pass the restriction by chance. The symbol \* indicates detected by Bluefuse™ also.

**Table 6.6.a:** Gene expression 4hr treated with  $\frac{1}{2}$  MIC ciprofloxacin normalised to time 0hr untreated control.

Gene Name	Normalised Expression	t-test <i>p</i> value	Rv No.	Predicted Function
Rv1985c*	0.31	1.54E-03	Rv1985c	transcriptional regulator (LysR family)
Rv0586*	0.32	6.84E-04	Rv0586	transcriptional regulator (GntR family)
<i>sigH</i> *	0.38	1.18E-04	Rv3223c	ECF subfamily sigma subunit
<i>end</i>	0.42	2.19E-03	Rv0670	endonuclease IV (apurinase)
<i>sigE</i>	0.51	1.84E-04	Rv1221	ECF subfamily sigma subunit
<i>rpoB</i>	0.64	4.95E-04	Rv0667	[beta] subunit of RNA polymerase
Rv2896c	1.68	1.89E-05	Rv2896c	conserved hypothetical protein
<i>dut</i>	1.89	5.34E-03	Rv2697c	deoxyuridine triphosphatase
Rv2821c	2.84	6.83E-05	Rv2821c	conserved hypothetical protein

**Table 6.6.b:** Gene expression 4hr treated with ¼ MIC ciprofloxacin normalised to time 0hr untreated control.

Gene Name	Normalised Expression	t-test <i>p</i> value	Rv No.	Predicted Function
Rv1985c	0.23	8.17E-07	Rv1985c	transcriptional regulator (LysR family)
Rv2817c	0.25	4.52E-10	Rv2817c	conserved hypothetical protein
Rv0938	0.45	1.82E-08	Rv0938	conserved hypothetical protein
Rv0586	0.47	2.73E-04	Rv0586	transcriptional regulator (GntR family)
<i>end</i>	0.50	3.35E-05	Rv0670	endonuclease IV (apurinase)
<i>sigE</i>	0.53	3.06E-04	Rv1221	ECF subfamily sigma subunit
<i>sigH</i>	0.55	9.09E-04	Rv3223c	ECF subfamily sigma subunit
Rv2816c	0.57	1.86E-05	Rv2816c	conserved hypothetical protein
<i>recF</i>	1.50	1.22E-03	Rv0003	DNA replication and SOS induction
<i>uvrD2</i>	1.51	3.40E-03	Rv3198c	putative UvrD
<i>recA</i>	1.78	5.16E-04	Rv2737c	recombinase (contains intein)
<i>dut</i>	2.00	1.99E-05	Rv2697c	deoxyuridine triphosphatase
Rv2896c	2.11	3.92E-03	Rv2896c	conserved hypothetical protein
<i>xthA</i> *	2.11	9.16E-06	Rv0427c	exodeoxyribonuclease III
Rv2821c*	2.27	7.26E-06	Rv2821c	conserved hypothetical protein

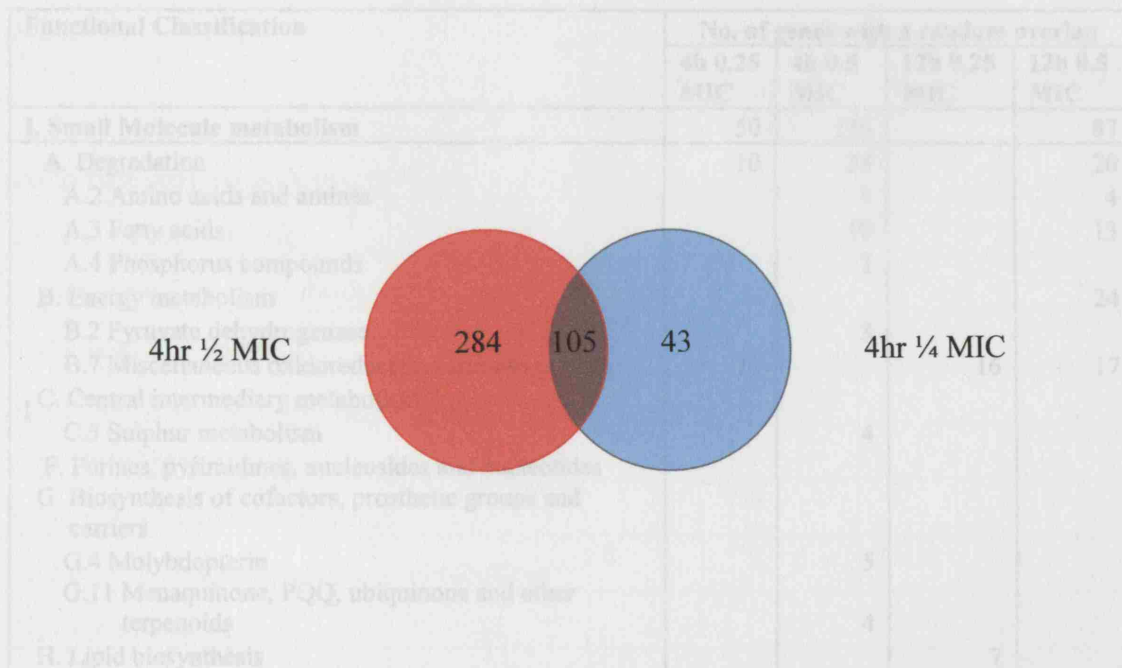
**Table 6.6.c:** Gene expression 12hr treated with ½ MIC ciprofloxacin normalised to time 0hr untreated control.

Gene Name	Normalised Expression	t-test <i>p</i> value	Rv No.	Predicted Function
<i>sigE*</i>	0.26	5.18E-03	Rv1221	ECF subfamily sigma subunit
<i>dinX</i>	0.42	2.18E-02	Rv1537	probable DNA-damage-inducible protein
Rv1956	0.56	2.71E-02	Rv1956	putative transcriptional regulator
<i>tagA</i>	1.44	3.10E-02	Rv1210	DNA-3-methyladenine glycosidase I
Rv3202c	1.51	6.95E-03	Rv3202c	Possible ATP-dependent DNA helicase
<i>nei</i>	1.60	1.66E-02	Rv3297	probable endonuclease VIII
<i>uvrD2</i>	1.65	1.16E-02	Rv3198c	putative UvrD
<i>mutT4</i>	1.76	8.24E-04	Rv3908	conserved hypothetical protein
Rv2978c*	1.88	8.24E-03	Rv2978c	transposase
<i>radA</i>	2.14	1.24E-02	Rv3585	probable DNA repair RadA homologue
Rv2791c	2.67	2.88E-02	Rv2791c	transposase
<i>dnaE1</i>	2.72	2.43E-03	Rv1547	DNA polymerase III, [alpha] subunit
<i>recA</i>	4.13	8.31E-06	Rv2737c	recombinase (contains intein)
<i>xthA</i>	4.30	1.33E-02	Rv0427c	exodeoxyribonuclease III

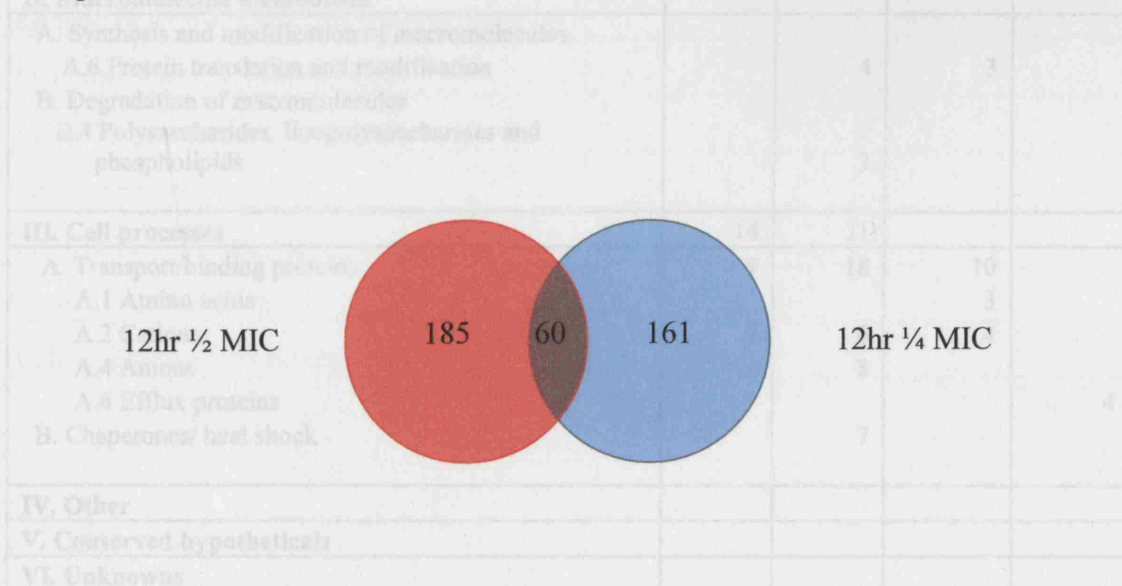
**Table 6.6.d:** Gene expression 12hr treated with ¼ MIC ciprofloxacin normalised to time 0hr untreated control.

Gene Name	Normalised Expression	t-test <i>p</i> value	Rv No.	Predicted Function
<i>sigH</i>	0.26	3.10E-03	Rv3223c	ECF subfamily sigma subunit
<i>sigE*</i>	0.27	3.29E-04	Rv1221	ECF subfamily sigma subunit
Rv1956	0.57	4.43E-03	Rv1956	putative transcriptional regulator
<i>dnaB</i>	1.66	2.79E-02	Rv0058	DNA helicase (contains intein)
<i>gyrA</i>	1.87	4.59E-03	Rv0006	DNA gyrase subunit A
<i>xthA</i>	3.13	1.96E-03	Rv0427c	exodeoxyribonuclease III

**Figure 6.1:** Venn diagram showing the overlap of genes identified by the Benjamini and Hochberg False Discovery Rate Analysis comparing the different treatments at the 4hr time point. Each gene list was identified as being differentially expressed compared to time 0hr.



**Figure 6.2:** Venn diagram showing the overlap of genes identified by the Benjamini and Hochberg False Discovery Rate Analysis comparing the different treatments at the 12hr time point. Each gene list was identified as being differentially expressed compared to time 0hr.



**Table 6.7:** The response of *M. tuberculosis* after 4hr and 12hr treatment with  $\frac{1}{2}$  and  $\frac{1}{4}$  MIC ciprofloxacin examined by functional category, as defined by Cole et al 1998.

All genes identified have random overlap *p*-values of <0.1 versus pathway.

Functional Classification	No. of genes with a random overlap			
	4h 0.25 MIC	4h 0.5 MIC	12h 0.25 MIC	12h 0.5 MIC
<b>I. Small Molecule metabolism</b>	50	136		87
A. Degradation	10	28		20
A.2 Amino acids and amines		5		4
A.3 Fatty acids		19		13
A.4 Phosphorus compounds		3		
B. Energy metabolism				24
B.2 Pyruvate dehydrogenase		3		
B.7 Miscellaneous oxidoreductases and oxygenases	11		16	17
C. Central intermediary metabolism				
C.5 Sulphur metabolism		4		
F. Purines, pyrimidines, nucleosides and nucleotides				
G. Biosynthesis of cofactors, prosthetic groups and carriers				
G.4 Molybdopterin		5		
G.11 Menaquinone, PQQ, ubiquinone and other terpenoids		4		
H. Lipid biosynthesis			7	
H.1 Synthesis of fatty and mycolic acids			4	
H.3 Acyltransferases, mycoloyltransferases and phospholipid synthesis				4
J. Broad regulatory functions	11	34		24
J.1 Repressors or activators		28		21
<b>II. Macromolecule metabolism</b>				
A. Synthesis and modification of macromolecules				
A.6 Protein translation and modification		4	3	
B. Degradation of macromolecules				
B.4 Polysaccharides, lipopolysaccharides and phospholipids		3		
<b>III. Cell processes</b>	14	31		
A. Transport/binding proteins	9	18	10	
A.1 Amino acids			3	
A.2 Cations	4	6	4	
A.4 Anions		8		
A.6 Efflux proteins				4
B. Chaperones/ heat shock		7		
<b>IV. Other</b>				
<b>V. Conserved hypotheticals</b>				
<b>VI. Unknowns</b>				



**Table 6.8:** The response of *M. tuberculosis* after 4hr and 12hr treatment with  $\frac{1}{2}$  and  $\frac{1}{4}$  MIC ciprofloxacin classified by pathway. All genes identified have random overlap *p*-values of <0.1 versus pathway.

Pathway	No. of genes with a random overlap			
	4h 0.25 MIC	4h 0.5 MIC	12h 0.25 MIC	12h 0.5 MIC
Ascorbate and aldarate metabolism			4	
Butanoate metabolism	7		8	
Cyanoamino acid metabolism				3
Fatty acid biosynthesis			4	
Flavonoids, stilbene and lignin biosynthesis	3			
Glutamate metabolism				4
Glycolysis / Gluconeogenesis		6		
Glyoxylate and dicarboxylate metabolism	3	5	3	3
Nitrogen metabolism				5
Peptidoglycan biosynthesis		3		3
Phenylalanine metabolism			5	
Propanoate metabolism		11		8
Pyruvate metabolism			5	
Reductive carboxylate cycle (CO <sub>2</sub> fixation)		5		
Selenoamino acid metabolism			4	
Sterol biosynthesis	3	4		
Sulphur metabolism			3	
Terpenoid biosynthesis		4		
Tyrosine metabolism	3		4	
Valine, leucine and isoleucine degradation		10		6

**Table 6.9:** The mean relative fold change and the standard error of the mean (S.E.M.) of 2 biological replicates, calculated by the equation  $2^{-\Delta\Delta C_T}$ , demonstrating the expression of *dnaE2* normalised to *sigA* after treatment with  $\frac{1}{4}$  and  $\frac{1}{2}$  MIC ciprofloxacin over time.

Time (hr)	Mean ( $\pm$ S.E.M.) Fold Change		
	No treatment	$\frac{1}{4}$ MIC	$\frac{1}{2}$ MIC
0	0.029 $\pm$ 0.015	--	--
4	--	0.17 $\pm$ 0.14	0.017 $\pm$ 0.004
12	--	0.09 $\pm$ 0.068	0.052 $\pm$ 0.003

## 6.4 Discussion

### 6.4.1 Gene expression pattern

Treatment with  $\frac{1}{2}$  and  $\frac{1}{4}$  MIC ciprofloxacin resulted in induction of genes associated with DNA repair and protection together with a range of non-specific effects. Genes involved in other stress responses due to hypoxic conditions, high temperature, starvation, among others were also identified in this system. Specific responses were recorded in genes responsible for DNA repair and protection (table 6.6.a-d).

### 6.4.2 Regulation of DNA repair genes

Genes directly involved in SOS repair and mutagenesis were investigated (table 6.2). Rapid up-regulation of *recA* up to 2-fold was observed at 4hr treatment with  $\frac{1}{4}$  MIC and 4 fold at 12hr treatment with  $\frac{1}{2}$  MIC ciprofloxacin indicating induction of the SOS response (table 6.6.b and 6.6.c, section 1.13.5.a SOS response). *recA* was also detected as being significantly expressed by the more stringent Bonferroni multiple testing correction. Briefly, RecA binds to regions of single stranded DNA which have accumulated when DNA is damaged, or when replication of DNA is blocked, and sets off a cascade of reactions (Ogawa & Ogawa 1990). Under normal cellular conditions the transcription of SOS genes are repressed. This is mediated by LexA binding to the SOS box, a specific sequence upstream of *lexA* and *recA* and all other genes belonging to the SOS system. However differences in *lexA* expression were not identified in any of the analysis performed during treatment. This might be because small changes in the expression of this gene are sufficient to affect binding to the LexA binding site. The level of expression of this gene may remain static because of

the organism's constant need for the SOS response. Small changes in LexA have been observed after various DNA damaging treatments but only after extended periods of exposure (Papavinasundaram et al. 2001). This raises the possibility that other factors might be involved in *recA* induction as relying on LexA cleavage alone might not result in sufficient induction. *recF*, involved in the assembly and disassembly of RecA filaments, responds to DNA damage (Davis et al. 2002). It is required for SOS induction by DNA damaging agents that produce single stranded lesions (McPartland, Green, & Echols 1980). The expression of *recF*, which modulates the assembly and disassembly of RecA filaments, was up-regulated 1.5 fold at 4h of treatment (table 6.6.b). Up-regulation of *uvrD2* was observed after 12hr treatment with  $\frac{1}{2}$  MIC ciprofloxacin (table 6.6.c). This helicase protein is involved in excision repair and recombination. Rv3202c, which has an UvrD domain, was found to be induced under the same conditions as *uvrD2*. *dnaB*, encodes a DNA helicase and was up-regulated 1.7 fold following  $\frac{1}{4}$  MIC treatment for 12hr. Rv3908, denoted *mutT4*, was up-regulated 1.8 fold at 12hr of  $\frac{1}{2}$  MIC treatment (table 6.6.c). It belongs to the family of MutT proteins which are anti-mutators involved in removing oxidative damaged nucleotides. Gene polymorphisms have been identified in the Beijing family of strains which are likely to result in a mutator phenotype and therefore lead to a better adaptation of the bacilli to a hostile environment (Rad et al. 2003). Up-regulation of *dut* which encodes a dUTPase was observed at 4hr in both treatments (table 6.6.a and 6.6.b). This product also enables the removal of damaged nucleotides.

Genes involved in base excision repair were induced. The expression of *xthA* was induced by 2 fold at 4hr and 4 fold at 12hr when compared to no treatment (table 6.6.b-d). This gene product, exodeoxyribonuclease III, is primarily involved in

correcting DNA damage as a result of exposure to reactive oxygen intermediates and other metabolites that oxidize and alkylate the DNA. At 12hr treatment with ½ MIC ciprofloxacin, *tagA* was induced (table 6.6.c). The product is DNA-3-methyladenine glycosidase I, which is involved in DNA repair. *end*, which encodes an endonuclease IV (apurinase), involved in base excision repair was significantly down-regulated at the 4hr time point. The expression of *ada*, *alkA*, *fpg*, *mpg*, *mutY*, *nth* and *ung*, all involved in base excision repair were not significantly different when normalized to untreated control.

*dnaE1* was significantly up-regulated by ciprofloxacin treatment (table 6.6.c). This encodes the alpha subunit of the major replicative DNA polymerase DnaE. This gene is thought to be essential for chromosomal replication as it cannot be inactivated in *M. tuberculosis* and *M. smegmatis* (Boshoff et al. 2003). Boshoff et al postulate that it is *dnaE2*, encodes the alpha chain of DNA polymerase III, which is involved in error prone repair leading to mutagenesis and ultimately the emergence of drug resistance. However expression of *dnaE2* was found unchanged in our system. The quality of the spot was checked manually on the array and it was flagged as empty which means the spot intensity is slightly above the background. The threshold for spot flagging was set to 1, the background mean is subtracted from the signal mean and this value is divided by the background standard deviation. The spot is flagged as empty if this ratio is less than 1.

Two fold induction of *radA* was observed at 12hr after ½ MIC treatment (table 6.6.c). This gene was one of the highest induced genes involved in DNA modification after

exposure to UV irradiation, mitomycin C and hydrogen peroxide (Boshoff et al. 2003). It encodes the DNA repair protein RadA.

Expression of Rv0938 was down-regulated and its product functions as a DNA ligase by sealing nicks in double stranded DNA during replication, recombination and repair. The DNA repair gene *dinX* which encodes a DNA damage inducible protein was down-regulated after 12hr ½ MIC treatment.

There was down-regulation among other sigma factors *sigE* and *sigH* under all conditions (table 6.6). Drug specific effects were observed as there was an up-regulation in *gyrA* of 1.9 fold after 12hr ½ MIC treatment. This gene encodes DNA gyrase which is the target of ciprofloxacin. The doses used in this experiment did not result in a reduction of numbers of viable bacteria (table 6.3).

#### **6.4.3 Genes identified from other expression profiling studies**

Genes identified from other expression studies as being differentially expressed under various stresses (antibiotic treatment, low oxygen, acid and heat shock and nutrient depletion) were also identified in this study.

Gene clusters (GC) identified from a study investigating the metabolic response to inhibitors of gyrase functions, novobiocin, ofloxacin and levofloxacin identified the up-regulation of SOS gene clusters (GC56, -126 and -137) as well as GC49 in novobiocin treatment (Boshoff et al. 2004). When comparing the data presented here that of Boshoff et al, 6/16 genes were induced in GC56 [Rv0184, Rv2737c (*recA*),

Rv2791c, Rv2978c, Rv3074, Rv3585 (*radA*)] and 1 gene down-regulated Rv3827. In GC126 1/8 (Rv0094c) genes were down-regulated. In GC137, no genes were identified as being differentially expressed. Three genes in GC49 were down-regulated [Rv0140, Rv0384c (*clp*) and Rv3054c]. It must be noted that these gene clusters were identified after treatment of 5 X MIC levofloxacin and 5-10 X MIC of ofloxacin. Different quinolones were used at greater than the MIC, so it is not possible to extrapolate to the system described here.

Rand et al identified inducible genes following mitomycin C (MMC) treatment in both wild type *M. tuberculosis* and a *recA* deletion strain using microarrays (Rand et al. 2003). Comparing the data in this study to Rand et al, some genes concur. Rv3777 and Rv3074 were up-regulated 1.9 fold after 12h ¼ MIC ciprofloxacin treatment and were identified as being induced by MMC in the wild type but not in the *recA* mutant. Rv3777 and Rv3074 encode a conserved hypothetical protein and a 3-hydroxyacyl-CoA-dehydrogenase respectively. An SOS box motif has been identified upstream of Rv3074. Rv3777 is co-transcribed with a gene having an SOS box (Rand et al. 2003). *dnaB* and *recA* were up-regulated in my study and were also induced by MMC. Both genes have an SOS box motif upstream which was shown to bind to LexA (Rand et al. 2003). *ahpC*, *rpII*, Rv2735c, Rv2791c, Rv2978c, Rv3263, *trxC*, *uvrD2* and *xthA* were found up-regulated in both studies. *leuC*, *lldD1*, *phoH2*, *pqqE*, Rv0823c, Rv2817c, Rv2989 and Rv3093c were found to be repressed in both studies. Overall the levels of induction and repression were greater following MMC than ciprofloxacin treatment.

Waddell et al have previously described defensive pathways for *M. tuberculosis* under treatment with isoniazid, isoxyl, tetrahydrolipstatin, and other agents at MIC levels, in

this study  $\frac{1}{2}$  and  $\frac{1}{4}$  MIC levels of a quinolone were used but the same gene groups were up-regulated (Waddell et al. 2004). Genes involved in efflux and transport such as *narK2* (nitrite extrusion protein), *ctpF* (probable cation transport ATPase), *efpA* (putative efflux protein) and *ctpC* (a probable metal cation transporter) and another subset of ESAT-6 proteins Rv1198, Rv1793 and Rv3874 were induced in both systems (table 6.8 Class III.A). Changes in metabolism seen under stress conditions were observed with an 8 fold overexpression of *gltA1* at 4hr (citrate synthase III) (McKinney et al. 2002). Rv1131 (*gltA1*) catalyzes the end product of the glyoxylate pathway (citrate) from acetyl coenzyme A (CoA) and oxaloacetate (table 6.9). Six fold upregulation of the upstream gene Rv1130, was observed. This is a conserved hypothetical protein which is homologous to *prpD* in *S. typhimurium* required for propanoate metabolism. Rv1130 is likely to encode 2-methylcitrate hydratase in *M. tuberculosis* (Horswill & Escalante-Semerena 2001). All of these genes are involved in fatty acid metabolism (6.7 Class IH). Rv1130 and Rv1131 (*gltA1*) are likely to be co-transcribed and form part of a pathway along with *accD3*, which encodes the acetyl/propionyl CoA carboxylase [beta] subunit (Bacon et al. 2004). This gene was up-regulated at 4hr and 12hr. These genes, all likely to be involved in propionate metabolism, have been identified in low oxygen tension, acid shock exposure to SDS and in macrophage infection (Fisher, Plikaytis, & Shinnick 2002; Horswill & Escalante-Semerena 2001; Textor et al. 1997). Another enzyme, besides *gltA1* as mentioned, involved in the glyoxylate pathway is *aceA* was induced (table 6.8 Class IB). This enzyme encodes isocitrate lyase which reversibly catalyses the conversion of isocitrate to succinate and glyoxylate. In H37Rv *aceA* is frameshifted and thought not to be functional (Honer Zu Bentrup et al. 1999).



Other genes known to be up-regulated in bacteria from previous studies under stress were induced in this system also. Induction of *sodA*, superoxide dismutase, destroys radicals which are normally produced within cells and are toxic to biological systems, was observed (table 6.8 Class IF). Overexpression of proteins involved in heat shock, Rv0251c (*hsp*), Rv0250c (Hsp20 family) and *hspG* (Hsp90 family) were identified (table 6.8 Class IIIB). The accumulation of unfolded polypeptides generated from a range of stress conditions is the likely trigger for the heat shock response (Narberhaus 1999). Upregulation of *grpE* which stimulates DnaK ATPase activity was observed. This gene is located in the major Hsp70 regulon consisting of DnaK, DnaJ and GrpE which are all regulated by the *hspR* repressor (Stewart, Robertson, & Young 2004). Chaperone proteins *groEL2*, *groES*, that prevent misfolding and promote refolding and assembly of unfolded polypeptides generated under stress conditions were induced. Genes involved in oxidative stress response *ahpC*, (alkyl hydroperoxide reductase) and *ahpD* (member of AhpC/TSA family) were up-regulated. *clpP* and *clpP2*, housekeeping genes were overexpressed at 4hr. *clp* has been identified in *E. coli* to cleave peptides in various proteins in processes requiring ATP hydrolysis. *nrdB* and *nrdE* encode a class Ib ribonucleotide reductase which plays an essential role in the cycling of nucleotides in the cell were expressed. Genes significantly up-regulated under low oxygen tension in other systems were induced such as *mbtD* and *mbtF* involved in mycobactin synthesis (Bacon et al. 2004). Mycobactin synthesis may be triggered as part of a generalised stress response preparing the bacteria for a hostile, nutrient depleted environment (similar to what the bacilli would encounter *in vivo*). Induction of a resuscitation promoting factor, *rpfE*, was observed at 4hr and 12hr treatment.

#### 6.4.4 The effect of sub-inhibitory concentrations of antibiotics on gene expression

Other studies have investigated sub-inhibitory concentrations of antibiotics on gene expression. Goh et al measured transcription patterns by the luminescence of promoter-reporter constructs in *S. typhimurium* (Goh et al. 2002). They used sub-inhibitory levels of a wide variety of antibiotics, all with different modes of action and found a general response with activation and repression of gene transcription. The gyrase B inhibitor novobiocin did not induce any response unlike erythromycin and rifampicin. These responses were distinct from the direct effects of the individual antibiotics. Antibiotics possess other activities besides growth inhibition such as the ability to modulate bacterial metabolism (CIBA Foundation Symposium 1992). Goh et al showed that this occurred at low antibiotic concentrations. Studies have revealed that any stress or starvation can result in a bacterial response in a similar set of general stress response proteins (Chang, Smalley, & Conway 2002; Hengge-Aronis 2002; Novotna et al. 2003). The expression of many of these genes are controlled by sigma factors (Wu, de Lencastre, & Tomasz 1996). Antibiotic exposure can also affect the control elements of the bacterial cell such as transcriptional activators (Barbosa & Levy 2000). Some belonging to the AraC family, Rv3833; the MerR family, Rv1994c, Rv3334 were expressed in this experiment. The gene *whiB7*, a putative transcriptional activator, has been shown to be induced at sub-inhibitory concentrations of antibiotics (Morris et al. 2005), was undetected in my study as being differentially expressed.

#### 6.4.5 General cellular responses

Genes involved in cell processes, small molecule and macromolecule metabolism were expressed (table 6.8). The largest group expressed was those involved in broad regulatory functions, specifically repressors or activators and in the pathway of propanoate metabolism (table 6.9).

Genes involved in ribosomal protein synthesis were up-regulated; 30S ribosomal proteins *rpsC* (S3), *rpsE* (S5), *rpsK* (S11), *rpsP* (S16), *rpsS* (S19) and 50S ribosomal proteins *rplF* (L6), *rplI* (L9), *rplP* (L16), *rplQ* (L17), *rplU* (L21), *rplW* (L23), *rplY* (L25). Four transcriptional regulators Rv0348, Rv3678c, Rv0043c and Rv1129c were up-regulated. This may indicate that there is no reduction in protein synthesis during treatment with ciprofloxacin. Thirty eight genes were down-regulated, including Rv1985c (regulatory protein LysR family) and Rv0586 (regulatory protein GntR family) which were involved in regulation. Rv0792c was the most down-regulated gene at 12h treatment with  $\frac{1}{4}$  MIC.

Changes were observed in genes involved in transposition of insertion elements. There was an induction of Rv1199c (possible IS1081 transposase), Rv2791c (IS1602 transposase) and Rv2978c (IS1538 transposase). Rv3827c, Rv3430c (IS1540 transposase), Rv2480c (possible IS6110 transposase) and Rv2649 (possible IS6110 transposase) were down-regulated.

Genes involved in cation transport as ATPases; *ctpC*, *ctpF*, *ctpG*, *ctpV* and *ftsQ* (ingrowth of wall at septum), *ftsW* (membrane protein) were down-regulated compared to time 0hr (table 6.8 Class III.A).

#### 6.4.6 Data analysis

The data was investigated in 2 ways, with Bluefuse™ technology or with ImaGene™ software followed by MAVI analysis and then entered into GeneSpring™. There was a difference in the number of genes identified as being significantly expressed between both types of analyses. This may be due to the increased stringency of the Bluefuse™ technology which uses only one scan per channel below saturation as opposed to ImaGene™ followed by MAVI in which 6 scans are analysed for each array taking into account scans at higher gain values where spots might be flagged incorrectly due to high background noise. The ANOVA based method Benjamini and Hochberg False Discovery Rate was used which estimates variance contributions common to all genes. ANOVA is particularly suited for estimating the amount of variation in several experimental steps such as mRNA preparation. The False Discovery Rate correction controls the portion of statistically significant expression differences that are false positives. This method ranks the *p*-values of each gene, multiplies the total number of genes in the gene list by the gene with second largest *p*-value and divides it by its rank. If this figure is less than 0.05, then it is deemed significant. This is continued down the list from the gene with the third largest *p*-value and so on. The gene with the largest *p*-value remains as it is. It is less stringent than the Bonferroni correction which controls the probability of making one false positive in the entire set of tests (Nadon et al. 2001). For instance after 4hr treatment with ¼

MIC ciprofloxacin, 71 genes were identified as being down-regulated and 15 as being up-regulated using the Bonferroni correction compared to 108 genes down-regulated and 40 genes up-regulated with the Benjamini and Hochberg correction. This multiple testing correction provides a good balance between the limitations of the occurrence of false positives and the discovery of statistically significant genes (Agilent Technologies 2005).

Some of genes identified as having statistically significant different expression may have been false positives. Using the Benjamini and Hochberg False Discovery Rate restriction with a  $p$ -value of  $<0.05$ , 5% of genes identified would be expected to pass the restriction by chance. Testing the whole gene list (3924 genes), 127 genes were identified as being up-regulated after 4hrs of  $\frac{1}{2}$  MIC treatment, 6 genes may have been identified by chance.

For the validation of the microarray data, the expression of *dnaE2* was compared to *sigA* by qRT-PCR. The changes in the steady-state levels of *dnaE2* relative to *sigA* are measured to give a relative quantitation using the  $2^{-\Delta\Delta CT}$  method (Livak & Schmittgen 2001). Only comparative differences can be determined by this method because the absolute quantity of the internal standard is not known. This is sufficient as the fold change is informative irrespective of the absolute value. It is necessary to use static housekeeping genes as internal standards. It may be necessary in some cases to determine the absolute quantitation when one needs to know the absolute amount of mRNA sequence in a sample. *sigA* was used as the normalising gene as it is considered to be relatively constant under stress conditions (Kendall et al. 2004; Manganelli et al. 1999). It is considered to be the primary sigma factor essential

for cell survival (Doukhan et al. 1995; Lonetto, Gribskov, & Gross 1992). *dnaE2* was chosen as it is the polymerase that is involved in damage inducible mutagenesis in *M. tuberculosis* (Boshoff et al. 2003). Also it was unusual that *dnaE2* was not identified as being differentially expressed at any of the treatments so it required further investigation. From the qRT-PCR experiments, there was no significant change in the expression of *dnaE2* normalised to *sigA* after the sub-inhibitory MIC treatment (table 6.9).

There were relatively few genes induced in this experiment. Only 18/110 genes involved in DNA repair, mutagenesis and recombination were identified as being induced following sub-inhibitory exposure to ciprofloxacin. The activity of the antibiotic was checked by performing a bioassay (table 6.4), in case this was the reason behind the small number of genes induced. However this showed normal bactericidal activity within the QC range for *E. coli* ATCC 25922. Shifts of gene expression in the scale of less than 10 fold may not be detected by microarray based transcriptome analysis whereas changes in this scale are readily detected by qRT-PCR (Shi et al. 2005). Perhaps some of the changes in gene expression that occurred may have been below the level of sensitivity of the microarray. Future experiments would be to analyse the expression of more genes involved in the SOS response which were up-regulated by microarray technology and also those which would expect to have been induced using quantitative real time reverse transcription PCR.

Further experiments could also involve the determination of the mutation rate in *M. tuberculosis* H37Rv upon exposure to sub-inhibitory concentrations of ciprofloxacin. In effect a repeat of the experiment which has been previously performed in *M.*

*fortuitum* (Gillespie et al. 2005). It would also be interesting to observe if there was a difference in the mutation rate of a *lexA* or *recA* deletion strain and compare it to wild-type.

The number of biological replicates in the experiment could be increased to improve statistical tests. In this experiment there were 3 biological replicates at time 0hr, 3 at 4hr treatment with  $\frac{1}{4}$  MIC, 2 at 4hr with  $\frac{1}{2}$  MIC and single replicates for 12hr treatment with  $\frac{1}{4}$  and  $\frac{1}{2}$  MIC. There was insufficient RNA in the samples from the 12hr time point to analyse further replicates. Each biological replicate was then spotted twice on separate arrays. It is likely due to the number of replicates at 0h and 4hr  $\frac{1}{4}$  MIC treatment, the data is represented with more accuracy than the other time points.

## 6.5 Conclusion

Genome wide expression profiling was applied to sub-inhibitory ciprofloxacin treatment of *M. tuberculosis* to map the adaptability of the bacilli in response to stressful conditions. We have previously reported that this stressful treatment for the bacilli can lead to an increase in mutation rate, which may be due to an induction of the SOS response leading to mutagenesis. Sub-inhibitory concentrations of ciprofloxacin can modulate transcription in *M. tuberculosis*. The results revealed significant changes in the expression of genes involved in DNA protection, repair and recombination as well as the generalised effects of a cell under stress. In order to observe greater changes in gene expression occurring in more genes involved in repair, recombination and mutagenesis, future experiments could involve analysing more DNA repair genes using quantitative real time reverse transcription PCR. Although DNA repair is activated by the stress of fluoroquinolone treatment, not all mutations are corrected resulting in the development of resistance. More DNA repair responses may have been seen after longer treatment. The slow response of *M. tuberculosis* to DNA damage may be beneficial in withstanding the defenses of the macrophage by sustaining an adaptive response over a longer period of time.



**Chapter 7: Final Discussion**

Drug resistance in *M. tuberculosis* occurs through genetic alterations in the genome. These are believed to be driven by random spontaneous mutation which occur at very low frequency (Riska, Jacobs, Jr., & Alland 2000). Inadequate therapy may favour the maintenance of a resistant phenotype by providing a selective growth advantage (Iseman 1993). Multiple drug resistance occurs as a result of exposure of the bacilli to monotherapy and remains a risk to global control of tuberculosis. Globally tuberculosis kills 2 million people annually, with 1500 people dying daily in Africa. The threat to public health has increased due to the emergence of antibiotic resistance. There has been no improvement in the treatment of tuberculosis, even though this situation is changing with promising compounds in the clinical trial and developmental stage (WHO 2006). However the standard regimen remains at 6 months treatment of three or four drugs.

Monotherapy can occur as a result of the penetrating ability of anti-tubercular agents into the different compartments where the bacteria reside. It is thought that the bacilli exist in these compartments in different physiological states where they have different susceptibilities to compounds (Hu & Coates 2003). It is suggested that drug resistance will only emerge if there are protected compartments or if the patient is non-compliant (Lipsitch & Levin 1998). Exposure to single agents can occur through poor absorption or the development of resistance to the other agents used in treatment. We have investigated alternative mechanisms by which mutation may occur; due to error prone DNA repair mechanisms brought about by sub-optimal dosing, the generation of reactive oxygen intermediates from environmental pressures such as oxidative stress

or through pre-existing sub-populations of resistant bacteria becoming fixed within the population. A further understanding of the development of resistance in *M. tuberculosis* is vital to determine what factors lead to the fixation of resistance in the population and will ultimately predict the likelihood that MDR tuberculosis will be a major health problem in the future.

Global health problems have developed from the emergence of drug resistant pathogens: drug resistant malaria from infection with *Plasmodium falciparum* developing resistance to chloroquine and sulfadoxine-pyrimethamine (Peters 1987) and the development of methicillin resistant *Staphylococcus aureus* which is endemic in most hospitals in the world and has the potential to be vancomycin resistant (Fluit et al. 2001). So the threat of the fixation of MDR-TB phenotype in the population is serious.

Mutation is a continuous process which occurs without the selective pressure of antibiotics and is essential for evolutionary diversity. The phenomenon of heteroresistance, the presence of antibiotic resistant and sensitive organisms within a population prior to drug exposure, has been investigated in clinical *M. tuberculosis* isolates (Rinder, Mieskes, & Loscher 2001). In chapter 3, populations of resistant bacteria and sensitive bacteria were detected in clinical isolates and laboratory strains of *M. fortuitum* and *M. smegmatis*. No genetic determinants of heteroresistance were determined but further work is needed. Evidence suggests that heteroresistant isolates are more likely to generate homogenous resistant isolates so the significance of this phenomenon in a clinical setting is important (Hiramatsu et al. 1997). The presence of a heterogeneous strain is not commonly detected in patient samples perhaps due to the

failure of primary cultivation techniques in identifying the slower growing bacterial population which occur as a result of reduction in viability from the acquisition of resistance being outgrown by the susceptible population (Billington, McHugh, & Gillespie 1999).

It has been proposed that the acquisition of drug resistance is associated with loss of virulence due to an accumulation of chromosomal point mutations which carry a fitness cost. Therefore a drug resistant strain is less able to compete with a drug sensitive strain. In a clinical setting with effective TB control, drug resistant *M. tuberculosis* strains, which were at least resistant to isoniazid, resulted in fewer secondary cases than drug susceptible strains (Burgos et al. 2003). This shows that strains which were isoniazid resistant are not likely to produce new drug resistant TB cases so isoniazid resistance results in fitness disadvantage and a reduction in virulence. This has been demonstrated in the animal models of tuberculosis (Cohn, Kovitz, & Oda 1954; Middlebrook & Cohn 1953; Mitchison et al. 1960). Davies et al. found that a multi-drug resistant isolate of *M. tuberculosis* had decreased fitness compared to a drug sensitive isolate (Davies et al. 2000). A range of virulence, some growing faster and some slower than the control strain, was demonstrated in a mouse model infected with strains resistant to various anti-tubercular agents (Ordway et al. 1995). It is also apparent that accumulation of resistance may not result in a physiological cost for the bacteria. Isoniazid resistant *M. tuberculosis* does not revert back to sensitivity despite the apparent virulence advantage. The mathematical model of Dye and Williams assumes that the sequence of mutations leading to multiple resistance in a bacterium would reduce its “fitness” or virulence (Dye & Williams 2000). This model is limited only to epidemiological data and does not take into

account the bacterial genotype. If this assumption were to be correct then a low ratio of disease to infection would lead to a decline in the prevalence of initial cases of MDR-TB.

Billington et al showed that the acquisition of mutations in *rpoB* are associated with a reduction in fitness for the strain *in vitro* (Billington, McHugh, & Gillespie 1999). The *rpoB* mutation isolated most frequently clinically (Ser531Leu) confers a less severe fitness deficit compared to other mutations. However the Ser531Leu mutation was not associated with a higher mutation rate. The fixation of strains which result in a modest fitness loss may predominate in the environment that is in the absence of spontaneous reversion.

The W-Beijing strain of *M. tuberculosis* is associated with antibiotic resistance and is widely disseminated (Werngren & Hoffner 2003). Some strains of this genotype have reduced growth compared to susceptible strains while others retained their virulence (Toungousova et al. 2004). The *in vitro* fitness of strains with the common mutation at Ser531Leu in *rpoB* did not change when compared to susceptible strains. Different mutations in *rpoB* had an improved growth rate, so it is likely that compensatory mutations were occurring to restore the bacteria's capacity for growth to varying degrees. It is likely that the wide dissemination of the W-Beijing genotype is because some of the strains harbour rifampicin mutations which result in the least physiological cost to the bacteria alongside compensatory mutations which restore fitness and not because of its ability to mutate. It has been shown that the Beijing genotype does not have an increased mutation rate (Werngren & Hoffner 2003).

*M. tuberculosis* is an intracellular pathogen which can survive adverse environments, with an ability to adapt to these conditions and persist in a dormant state within the human host which can reactivate to cause disease. It must protect itself against reactive oxygen intermediates generated by metabolism. The hypothesis that oxidative stress is the driving force of mutations in *M. tuberculosis* was investigated in chapter 4. Reactive oxygen intermediates damage DNA, causing the oxidative of guanine, forming a GO lesion and causing G·C → T·A mutations (Jackett, Aber, & Lowrie 1978) or through the deamination of cytosine resulting in G·C → A·T changes (Purmal, Kow, & Wallace 1994a; Purmal, Kow, & Wallace 1994b). Mutations in *rpoB* and *pncA* should occur after isoniazid resistance mutations, which are thought to reduce an organism's capacity to deal with oxidative stress. There was evidence of this damage in the high occurrence of C → T changes in *rpoB* which may have been driven by oxidised cytosine due to reactive oxygen intermediates. However, an equally high number of G → A changes on the opposite strand would have been expected; this was not the case. This mutation pattern was not observed in *pncA*. G·C → T·A mutations occurred in low frequencies in both genes, suggesting that *M. tuberculosis* is competent in repair of oxidised guanine via the MutM/MutY pathway. This would lead one to conclude that it is the relative fitness of a strain which leads to mutation fixation. Previous studies have shown that acquisition of resistance has resulted in a reduction in fitness. Furthermore the mutants with the greatest selection *in vivo* are those which confer the least fitness deficit. On the part of resistant bacteria, the loss of biological fitness may easily be overcome by the acquisition of compensatory mutations, thereby stabilizing the resistant bacteria within a given population (Bottger et al. 1998). Compensatory mutations are likely to occur enabling the adaptation of the bacteria to the loss in fitness. These mutations could occur

throughout the genome randomly allowing a bacterial population to maintain its genes intact, while increasing its genetic variability under stress.

The hypothesis that an isoniazid resistant strain with pre-existing mutations in *katG*, rendering it deficient in protection against anti-oxidants, generates a hypermutator phenotype was investigated in chapter 5. The presence of a hypermutator strain could explain the onset of multiple drug resistance. The increase in mutation was investigated upon exposure to oxidative stress. No differences were detected in the mutation rate of this mutant strains when they were treated with hydrogen peroxide, regardless if there was detectable catalase-peroxidase activity (Pym, Saint-Joanis, & Cole 2002). This suggests that the bacteria are adapting to their stressful environment through compensatory mutations. Overexpression of *ahpC* among isoniazid resistant strains with a mutation in *katG* may provide an alternative mechanism of protection against antioxidants (Guimaraes et al. 2005). Mutations occurring in the promoter region of this gene may compensate for the loss or reduction of catalase-peroxidase activity (Sherman et al. 1996). Upon screening of rifampicin resistant mutants, a completely different pattern of mutations were observed than expected. Only 3/66 colonies had mutations within the RRDR, 2 of these were the most frequently isolated Ser531Leu change. It has been observed in an isoniazid resistant outbreak in London that a different pattern of *rpoB* mutations occurred inside and outside the RRDR (Jenkins et al. 2005). These polymorphisms account for <8% of resistance mutations isolated clinically. All of the isolates came from a major epidemic in North London of isoniazid monoresistance identified by a single genotype (Ruddy et al. 2004). This would suggest that the development of rifampicin resistance depends on a complexity

of factors which ultimately reflects the ability of mycobacteria to adapt to its environment in the host.

Drug resistant strains which result in a minimal fitness cost may become fixed in the population. This is the case for isoniazid resistant *M. tuberculosis* strains which have the *katG* Ser315Thr mutation. Pym et al found that this strain retained catalase-peroxidase activity and was virulent in the mouse model (Pym, Saint-Joanis, & Cole 2002). This mutation did not result in a significant loss of bacterial fitness. Usually the acquisition of drug resistance is detrimental for the bacteria resulting in a loss of biological fitness (Gillespie 2001). However, this fitness deficit among resistant strains can be overcome by compensatory mutations which will then stabilise the resistant bacteria within the population (Lenski 1998). In streptomycin resistant *E. coli* harbouring mutations in *rpsL*, second site mutations compensate for the original substantial fitness cost by 14-18% per generation (Schrag, Perrot, & Levin 1997). Reversion to streptomycin sensitivity conferred a strong selective disadvantage (4-30% per generation) relative to adapted resistant strains.

Genetic diversity is generated by endogenous mutagenesis at a cellular level which is controlled by the SOS response in bacteria. The response of the bacteria ensures that fitness is competitive by enhancing survival. *M. tuberculosis* has a competent DNA repair system which can deal with the mutagenic effects exerted by the immune system (Flynn & Chan 2001). *M. tuberculosis* lacks homologues of a mismatch repair system which play a role in genome stability and mutation avoidance (Mizrahi & Andersen 1998). So *M. tuberculosis* may be efficient at mismatch repair via

alternative pathways which could lead to mutation fixation as has been demonstrated in humans (Huang et al. 1994); repair by MutY or the UvrABC excinuclease.

The emergence of resistance in bacteria depends on the selective pressure of antibiotics so a direct correlation between antibiotic use and the development of bacterial resistance to antibiotics over periods of time can exist (Austin, Kristinsson, & Anderson 1999). Treatment with sub-inhibitory concentrations of quinolone have been shown to increase the mutation rate of *M. fortuitum* by up to 120 fold (Gillespie et al. 2005). It is known that quinolones are potent inducers of the SOS response (Phillips et al. 1987). So the likely cause of the increase in mutation is as a result of induction of the SOS response – which initiates error prone repair by DNA polymerases. This was investigated in chapter 6 using genetic profiling with whole genome arrays of *M. tuberculosis* to identify the activity of DNA repair genes under sub-MIC levels of quinolone. Differential expression of some genes involved in DNA repair were observed; *dut*, *radA*, *recA*, *recF*, *mutT4*, *nei*, *tagA*, *uvrD2* and *xthA*. However along with the induction of these genes there was also down-regulation of other DNA repair genes. From this work, one could conclude that the DNA repair genes were up and down regulated. The SOS response might be continually triggered in *M. tuberculosis* during infection through the presence of reactive nitrogen and oxygen intermediates produced by the immune system (Tippin, Pham, & Goodman 2004). This would mean that subtle changes undetectable by microarray technology may occur. qRT-PCR was performed to identify smaller fold changes in expression and validate the microarray data. Future work could involve the further use of qRT-PCR to investigate more genes involved in DNA repair, recombination and mutagenesis.



Therefore the development of multiple drug resistance depends on a multitude of factors; the ability of the strain to adapt to its host environment through compensatory mutations and competent repair mechanisms and the selection of a resistance allele which produces the least fitness deficit. The success of the mycobacteria in fixing these mutations will determine how serious the problem of MDR tuberculosis will be in the future.

## REFERENCES

Adams, L. B., Dinauer, M. C., Morgenstern, D. E., & Krahenbuhl, J. L. 1997, "Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to *Mycobacterium tuberculosis* using transgenic mice", *Tuber.Lung Dis.*, vol. 78, no. 5-6, pp. 237-246.

Agilent Technologies 2005, *Multiple Testing Corrections*.

Ahmad, S., Araj, G. F., Akbar, P. K., Fares, E., Chugh, T. D., & Mustafa, A. S. 2000, "Characterization of *rpoB* mutations in rifampin-resistant *Mycobacterium tuberculosis* isolates from the Middle East", *Diagnostic Microbiology and Infectious Disease*, vol. 38, no. 4, pp. 227-232.

Ahmad, S., Mokaddas, E., & Fares, E. 2002, "Characterization of *rpoB* mutations in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates from Kuwait and Dubai", *Diagn.Microbiol.Infect.Dis.*, vol. 44, no. 3, pp. 245-252.

Ainsa, J. A., Blokpoel, M. C., Otal, I., Young, D. B., De Smet, K. A., & Martin, C. 1998, "Molecular Cloning and Characterization of Tap, a Putative Multidrug Efflux Pump Present in *Mycobacterium fortuitum* and *Mycobacterium tuberculosis*", *The Journal of Bacteriology*, vol. 180, no. 22, pp. 5836-5843.

Akaki, T., Tomioka, H., Shimizu, T., Dekio, S., & Sato, K. 2000, "Comparative roles of free fatty acids with reactive nitrogen intermediates and reactive oxygen intermediates in expression of the anti-microbial activity of macrophages against *Mycobacterium tuberculosis*", *Clin.Exp.Immunol.*, vol. 121, no. 2, pp. 302-310.

Alam, M. R., Donabedian, S., Brown, W., Gordon, J., Chow, J. W., Zervos, M. J., & Hershberger, E. 2001, "Heteroresistance to vancomycin in *Enterococcus faecium*", *J.Clin.Microbiol.*, vol. 39, no. 9, pp. 3379-3381.

- Alland, D., Kalkut, G. E., Moss, A. R., McAdam, R. A., Hahn, J. A., Bosworth, W., Drucker, E., & Bloom, B. R. 1994, "Transmission of Tuberculosis in New York City -- An Analysis by DNA Fingerprinting and Conventional Epidemiologic Methods", *The New England Journal of Medicine*, vol. 330, no. 24, pp. 1710-1716.
- Allison, M. J., Zappasodi, P., & Lurie, M. B. 1962, "Host parasite relationships in natively resistant and susceptible rabbits on quantitative inhalation of tubercle bacilli. Their significance for the nature of genetic resistance", *Am.Rev.Respir.Dis.*, vol. 85, pp. 553-569.
- Altamirano, M., Marostenmaki, J., Wong, A., FitzGerald, M., Black, W. A., & Smith, J. A. 1994, "Mutations in the catalase-peroxidase gene from isoniazid-resistant *Mycobacterium tuberculosis* isolates", *J.Infect.Dis.*, vol. 169, no. 5, pp. 1162-1165.
- Altrichter, T. & Heizmann, W. R. 1994, "[Gardnerella vaginalis: transport, microscopy, testing resistance]", *Geburtshilfe Frauenheilkd.*, vol. 54, no. 11, pp. 606-611.
- Andersen, P., Askgaard, D., Ljungqvist, L., Bennedsen, J., & Heron, I. 1991, "Proteins released from *Mycobacterium tuberculosis* during growth", *Infection and Immunity*, vol. 59, no. 6, pp. 1905-1910.
- Andersson, D. I. & Levin, B. R. 1999, "The biological cost of antibiotic resistance", *Current Opinion in Microbiology*, vol. 2, no. 5, pp. 489-493.
- Armitage, P. 1952, "The statistical theory of bacterial populations subject to mutation", *J.R.Stat.Soc.B*, vol. 14, pp. 1-40.
- Arriaga-Alba, M., Rivera-Sanchez, R., Parra-Cervantes, G., Barro-Moreno, F., Flores-Paz, R., & Garcia-Jimenez, E. 2000, "Antimutagenesis of [beta]-Carotene to Mutations Induced by Quinolone on *Salmonella typhimurium*", *Archives of Medical Research*, vol. 31, no. 2, pp. 156-161.
- Arrigo, C. J., Singh, K., & Modak, M. J. 2002, "DNA Polymerase I of *Mycobacterium tuberculosis*. Functional role of a conserved aspartate in the hinge joining the m and n helices", *Journal of Biological Chemistry*, vol. 277, no. 3, pp. 1653-1661.

- Aubry, A., Pan, X. S., Fisher, L. M., Jarlier, V., & Cambau, E. 2004, "*Mycobacterium tuberculosis* DNA Gyrase: Interaction with Quinolones and Correlation with Antimycobacterial Drug Activity", *Antimicrobial Agents and Chemotherapy*, vol. 48, no. 4, pp. 1281-1288.
- Austin, D. J., Kristinsson, K. G., & Anderson, R. M. 1999, "The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance", *Proc.Natl.Acad.Sci.U.S.A*, vol. 96, pp. 1152-1156.
- Bacon, J., James, B. W., Wernisch, L., Williams, A., Morley, K. A., Hatch, G. J., Mangan, J. A., Hinds, J., Stoker, N. G., Butcher, P. D., & Marsh, P. D. 2004, "The influence of reduced oxygen availability on pathogenicity and gene expression in *Mycobacterium tuberculosis*", *Tuberculosis.(Edinb.)*, vol. 84, no. 3-4, pp. 205-217.
- Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., Collins, D., de Lisle, G., & Jacobs, W. R., Jr. 1994, "*inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*", *Science*, vol. 263, no. 5144, pp. 227-230.
- Banner, A. S. 1979, "Tuberculosis. Clinical aspects and diagnosis", *Arch.Intern.Med.*, vol. 139, no. 12, pp. 1387-1390.
- Barbe, J., Villaverde, A., & Guerrero, R. 1983, "Evolution of cellular ATP concentration after UV-mediated induction of SOS system in *Escherichia coli*", *Biochem.Biophys.Res.Commun.*, vol. 117, no. 2, pp. 556-561.
- Barbosa, T. M. & Levy, S. B. 2000, "Differential Expression of over 60 Chromosomal Genes in *Escherichia coli* by Constitutive Expression of MarA", *The Journal of Bacteriology*, vol. 182, no. 12, pp. 3467-3474.
- Barnard, F. M. & Maxwell, A. 2001, "Interaction between DNA Gyrase and Quinolones: Effects of Alanine Mutations at GyrA Subunit Residues Ser83 and Asp87", *Antimicrobial Agents and Chemotherapy*, vol. 45, no. 7, pp. 1994-2000.

- Barnes, P. F. 1994, "T-Cell Responses and Cytokines," in *Tuberculosis: Pathogenesis, Protection and Control*, B. R. Bloom, ed., American Society for Microbiology, Washington DC, pp. 417-435.
- Barnett, M., Busby, S. R., & Mitchison, D. A. 1953, "Tubercle bacilli resistant to isoniazid: virulence and response to treatment with isoniazid in guinea-pigs and mice", *Br.J.Exp.Pathol.*, vol. 34, no. 5, pp. 568-581.
- Bates, H., Randall, S. K., Rayssiguier, C., Bridges, B. A., Goodman, M. F., & Radman, M. 1989, "Spontaneous and UV-induced mutations in *Escherichia coli* K-12 strains with altered or absent DNA polymerase I", *The Journal of Bacteriology*, vol. 171, no. 5, pp. 2480-2484.
- Bemer, P., Palicova, F., Rusch-Gerdes, S., Drugeon, H. B., & Pfyffer, G. E. 2002, "Multicenter Evaluation of Fully Automated BACTEC Mycobacteria Growth Indicator Tube 960 System for Susceptibility Testing of *Mycobacterium tuberculosis*", *Journal of Clinical Microbiology*, vol. 40, no. 1, pp. 150-154.
- Berning, S. E. 2001, "The role of fluoroquinolones in tuberculosis today", *Drugs*, vol. 61, no. 1, pp. 9-18.
- Bertrand-Burggraf, E., Hurstel, S., Daune, M., & Schnarr, M. 1987, "Promoter properties and negative regulation of the *uvrA* gene by the LexA repressor and its amino-terminal DNA binding domain", *J.Mol.Biol.*, vol. 193, no. 2, pp. 293-302.
- Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A., & Duncan, K. 2002, "Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling", *Molecular Microbiology*, vol. 43, no. 3, pp. 717-731.
- Betts, J. C., McLaren, A., Lennon, M. G., Kelly, F. M., Lukey, P. T., Blakemore, S. J., & Duncan, K. 2003, "Signature Gene Expression Profiles Discriminate between Isoniazid-, Thiolactomycin-, and Triclosan-Treated *Mycobacterium tuberculosis*", *Antimicrobial Agents and Chemotherapy*, vol. 47, no. 9, pp. 2903-2913.

- Billington, O. J., McHugh, T. D., & Gillespie, S. H. 1999, "Physiological cost of rifampin resistance induced *in vitro* in *Mycobacterium tuberculosis*", *Antimicrobial Agents and Chemotherapy*, vol. 43, no. 8, pp. 1866-1869.
- Bisognano, C., Kelley, W. L., Estoppey, T., Francois, P., Schrenzel, J., Li, D., Lew, D. P., Hooper, D. C., Cheung, A. L., & Vaudaux, P. 2004, "A RecA-LexA-dependent Pathway Mediates Ciprofloxacin-induced Fibronectin Binding in *Staphylococcus aureus*", *Journal of Biological Chemistry*, vol. 279, no. 10, pp. 9064-9071.
- Bjorkman, J., Nagaev, I., Berg, O. G., Hughes, D., & Andersson, D. I. 2000, "Effects of Environment on Compensatory Mutations to Ameliorate Costs of Antibiotic Resistance", *Science*, vol. 287, no. 5457, pp. 1479-1482.
- Bjorkman, J., Hughes, D., & Andersson, D. I. 1998, "Virulence of antibiotic-resistant *Salmonella typhimurium*", *Proceedings of the National Academy of Sciences*, vol. 95, no. 7, pp. 3949-3953.
- Blanchard, J. S. 1996, "Molecular mechanisms of drug resistance in *Mycobacterium tuberculosis*", *Annu.Rev.Biochem.*, vol. 65, pp. 215-239.
- Blanche, F., Cameron, B., Bernard, F. X., Maton, L., Manse, B., Ferrero, L., Ratet, N., Lecoq, C., Goniot, A., Bisch, D., & Crouzet, J. 1996, "Differential behaviors of *Staphylococcus aureus* and *Escherichia coli* type II DNA topoisomerases", *Antimicrobial Agents and Chemotherapy*, vol. 40, no. 12, pp. 2714-2720.
- Bleiber, G., Munoz, M., Ciuffi, A., Meylan, P., & Telenti, A. 2001, "Individual contributions of mutant protease and reverse transcriptase to viral infectivity, replication, and protein maturation of antiretroviral drug resistant human immunodeficiency virus type 1", *Journal of Virology*, vol. 75, pp. 3291-3300.
- Bloom, B. R. & Fine, P. E. M. 1994, "The BCG experience: implications for future vaccines against tuberculosis.," in *Tuberculosis: Pathogenesis, Protection and Control.*, B. R. Bloom, ed., ASM Press, Washington DC, pp. 531-557.

- Bloom, B. R., Flynn, J., McDonough, K., Kress, Y., & Chan, J. 1994, "Experimental approaches to mechanisms of protection and pathogenesis in *M. tuberculosis* infection", *Immunobiology*, vol. 191, no. 4-5, pp. 526-536.
- Bloom, B. R. 2002, "Tuberculosis -- The Global View", *The New England Journal of Medicine*, vol. 346, no. 19, pp. 1434-1435.
- Blower, S. M., McLean, A. R., Porco, T. C., Small, P. M., Hopewell, P. C., Sanchez, M. A., & Moss, A. R. 1995, "The intrinsic transmission dynamics of tuberculosis epidemics", *Nat.Med.*, vol. 1, no. 8, pp. 815-821.
- Boiteux, S. & Laval, J. 1983, "Imidazole open ring 7-methylguanine: an inhibitor of DNA synthesis", *Biochemical and Biophysical Research Communications*, vol. 110, no. 2, pp. 552-558.
- Borgdorff, M. W., Behr, M. A., Nagelkerke, N. J., Hopewell, P. C., & Small, P. M. 2000, "Transmission of tuberculosis in San Francisco and its association with immigration and ethnicity", *Int.J Tuberc.Lung Dis.*, vol. 4, no. 4, pp. 287-294.
- Borgdorff, M. W., Nagelkerke, N., Van Soolingen, D., de Haas, P. E., Veen, J., & van Embden, J. D. 1998, "Analysis of tuberculosis transmission between nationalities in the Netherlands in the period 1993-1995 using DNA fingerprinting", *American Journal of Epidemiology*, vol. 147, no. 2, pp. 187-195.
- Boshoff, H. I. M., Myers, T. G., Copp, B. R., McNeil, M. R., Wilson, M. A., & Barry, C. E., III 2004, "The Transcriptional Responses of *Mycobacterium tuberculosis* to Inhibitors of Metabolism: Novel Insights Into Drug Mechanisms Of Action", *Journal of Biological Chemistry*, vol. 279, no. 38, pp. 40174-40184.
- Boshoff, H. I., Reed, M. B., Barry, C. E., III, & Mizrahi, V. 2003, "DnaE2 polymerase contributes to *in vivo* survival and the emergence of drug resistance in *Mycobacterium tuberculosis*", *Cell*, vol. 113, no. 2, pp. 183-193.

- Bottger, E. C., Springer, B., Pletschette, M., & Sander, P. 1998, "Fitness of antibiotic-resistant microorganisms and compensatory mutations", *Nat.Med.*, vol. 4, no. 12, pp. 1343-1344.
- Brawn, M. K. & Fridovich, I. 1985, "Increased superoxide radical production evokes inducible DNA repair in *Escherichia coli*", *Journal of Biological Chemistry*, vol. 260, no. 2, pp. 922-925.
- Breines, D. M., Ouabdesselam, S., Ng, E. Y., Tankovic, J., Shah, S., Soussy, C. J., & Hooper, D. C. 1997, "Quinolone resistance locus *nfxD* of *Escherichia coli* is a mutant allele of the *parE* gene encoding a subunit of topoisomerase IV", *Antimicrobial Agents and Chemotherapy*, vol. 41, no. 1, pp. 175-179.
- Brennan, P. J. 2003, "Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*", *Tuberculosis*, vol. 83, no. 1-3, pp. 91-97.
- Brent, R. & Ptashne, M. 1981, "Mechanism of action of the *lexA* gene product", *Proc.Natl.Acad.Sci.U.S.A*, vol. 78, no. 7, pp. 4204-4208.
- Brooks, P. C., Movahedzadeh, F., & Davis, E. O. 2001, "Identification of Some DNA Damage-Inducible Genes of *Mycobacterium tuberculosis*: Apparent Lack of Correlation with LexA Binding", *The Journal of Bacteriology*, vol. 183, no. 15, pp. 4459-4467.
- Brosch, R., Gordon, S. V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., Kremer, K., Parsons, L. M., Pym, A. S., Samper, S., Van Soolingen, D., & Cole, S. T. 2002, "A new evolutionary scenario for the *Mycobacterium tuberculosis* complex", *Proceedings of the National Academy of Sciences*, vol. 99, no. 6, pp. 3684-3689.
- Brosch, R., Gordon, S. V., Billault, A., Garnier, T., Eiglmeier, K., Soravito, C., Barrell, B. G., & Cole, S. T. 1998, "Use of a *Mycobacterium tuberculosis* H37Rv Bacterial Artificial Chromosome Library for Genome Mapping, Sequencing, and Comparative Genomics", *Infection and Immunity*, vol. 66, no. 5, pp. 2221-2229.



- Brown, T. J., Tansel, O., & French, G. L. 2000, "Simultaneous identification and typing of multi-drug-resistant *Mycobacterium tuberculosis* isolates by analysis of *pncA* and *rpoB*", *Journal of Medical Microbiology*, vol. 49, no. 7, pp. 651-656.
- Bucher, H. C., Griffith, L. E., Guyatt, G. H., Sudre, P., Naef, M., Sendi, P., & Battegay, M. 1999, "Isoniazid prophylaxis for tuberculosis in HIV infection: a meta-analysis of randomized controlled trials", *AIDS*, vol. 13, no. 4, pp. 501-507.
- Burgos, M., DeRiemer, K., Small, P. M., Hopewell, P. C., & Daley, C. L. 2003, "Effect of drug resistance on the generation of secondary cases of tuberculosis", *J Infect.Dis.*, vol. 188, no. 12, pp. 1878-1884.
- Butler, W. R. & Kilburn, J. O. 1983, "Susceptibility of *Mycobacterium tuberculosis* to pyrazinamide and its relationship to pyrazinamidase activity", *Antimicrobial Agents and Chemotherapy*, vol. 24, no. 4, pp. 600-601.
- Cairns, J., Overbaugh, J., & Miller, S. 1988, "The origin of mutants", *Nature*, vol. 335, pp. 142-145.
- Calmette, A. 1927, *La vaccination préventive contre la tuberculose*. Masson et cie, Paris.
- Camie, J., Christensen, A., Eyeson-Annan, M., Gill, J., Konstantinos, A., Krause, V., Hurwitz, M., & Misrachi, A. 2001, "Tuberculosis notifications in Australia, 1998", *Commun.Dis.Intell.*, vol. 25, no. 1, pp. 1-8.
- Camus, J. C., Pryor, M. J., Medigue, C., & Cole, S. T. 2002, "Re-annotation of the genome sequence of *Mycobacterium tuberculosis* H37Rv", *Microbiology*, vol. 148, no. Pt 10, pp. 2967-2973.
- Canci, A., Minozzi, S., & Borgognini Tarli, S. 1996, "New Evidence of Tuberculous Spondylitis from Neolithic Liguria (Italy)", *International Journal of Antimicrobial Agents*, vol. 6, pp. 497-501.
- Canetti, G. 1955, *The Tubercle Bacillus in the Pulmonary Lesion of Man* Springer Publishing Company, New York.

- Capizzi, R. L. & Jameson, J. W. 1973, "A table for the estimation of the spontaneous mutation rates of cells in culture", *Mutation Research*, vol. 17, pp. 147-148.
- Casaregola, S., D'Ari, R., & Huisman, O. 1982, "Role of DNA replication in the induction and turn-off of the SOS response in *Escherichia coli*", *Mol.Gen.Genet.*, vol. 185, no. 3, pp. 440-444.
- Cavusoglu, C., Hilmioglu, S., Guneri, S., & Bilgic, A. 2002, "Characterization of *rpoB* mutations in rifampin-resistant clinical isolates of *Mycobacterium tuberculosis* from Turkey by DNA sequencing and line probe assay", *Journal of Clinical Microbiology*, vol. 40, no. 12, pp. 4435-4438.
- CDC 2000, *Centres for Disease Control and Prevention. Reported tuberculosis in the United States. 2000. Atlanta, Georgia.*
- CDC 2004, *CDC. Reported Tuberculosis in the United States, 2003. Atlanta, GA: U.S. Department of Health and Human Services, CDC.*
- Celesk, R. A. & Robillard, N. J. 1989, "Factors influencing the accumulation of ciprofloxacin in *Pseudomonas aeruginosa*", *Antimicrobial Agents and Chemotherapy*, vol. 33, no. 11, pp. 1921-1926.
- Chakrabarti, S. L. & Gorini, L. 1975, "A Link between Streptomycin and Rifampicin Mutation", *Proceedings of the National Academy of Sciences*, vol. 72, no. 6, pp. 2084-2087.
- Chan, E. D. & Iseman, M. D. 2002, "Current medical treatment for tuberculosis", *BMJ*, vol. 325, no. 7375, pp. 1282-1286.
- Chang, D. E., Smalley, D. J., & Conway, T. 2002, "Gene expression profiling of *Escherichia coli* growth transitions: an expanded stringent response model", *Molecular Microbiology*, vol. 45, no. 2, pp. 289-306.
- Chaparas, S. D. 1982, "Immunity in tuberculosis", *Bull. World Health Organ*, vol. 60, no. 4, pp. 447-462.

## References

- Chaudhury, A. M. & Smith, G. R. 1985, "Role of *Escherichia coli* RecBC enzyme in SOS induction", *Mol.Gen.Genet.*, vol. 201, no. 3, pp. 525-528.
- Chen, C. R., Malik, M., Snyder, M., & Drlica, K. 1996, "DNA Gyrase and Topoisomerase IV on the Bacterial Chromosome: Quinolone-induced DNA Cleavage", *Journal of Molecular Biology*, vol. 258, no. 4, pp. 627-637.
- Chen, L., Xie, Q. W., & Nathan, C. 1998, "Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates", *Mol.Cell*, vol. 1, no. 6, pp. 795-805.
- Cheng, S. J., Thibert, L., Sanchez, T., Heifets, L., & Zhang, Y. 2000, "*pncA* mutations as a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*: spread of a monoresistant strain in Quebec, Canada", *Antimicrobial Agents and Chemotherapy*, vol. 44, no. 3, pp. 528-532.
- Choy, H. E. & Fowler, R. G. 1985, "The specificity of base-pair substitution induced by the *mutL* and *mutS* mutators in *E. coli*", *Mutat.Res.*, vol. 142, no. 3, pp. 93-97.
- Christman, M. F., Morgan, R. W., Jacobson, F. S., & Ames, B. N. 1985, "Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*", *Cell*, vol. 41, no. 3, pp. 753-762.
- CIBA Foundation Symposium 1992, *Secondary metabolites: their function and evolution Vol 171* John Wiley and Sons, Chichester.
- Cockerill, F. R., III, Uhl, J. R., Temesgen, Z., Zhang, Y., Stockman, L., Roberts, G. D., Williams, D. L., & Kline, B. C. 1995, "Rapid identification of a point mutation of the *Mycobacterium tuberculosis* catalase-peroxidase (*katG*) gene associated with isoniazid resistance", *J.Infect.Dis.*, vol. 171, no. 1, pp. 240-245.
- Cohn, M., Kovitz, C., & Oda, U. 1954, "Studies on isoniazid and tubercle bacilli: the growth requirements, catalase activities, and pathogenic properties of isoniazid resistant mutants", *Am.Rev.Tuberc.*, vol. 70, pp. 641-664.

- Cole, S. T. 2002, "Comparative and functional genomics of the *Mycobacterium tuberculosis* complex.", *Microbiology*, vol. 148, pp. 2919-2928.
- Cole, S. T. & Barrell, B. G. 1998, "Analysis of the genome of *Mycobacterium tuberculosis* H37Rv", *Novartis.Found.Symp.*, vol. 217, pp. 160-172.
- Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., III, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., & Barrell, B. G. 1998, "Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence", *Nature*, vol. 393, no. 6685, pp. 537-544.
- Cole, S. T., Eiglmeier, K., Parkhill, J., James, K. D., Thomson, N. R., Wheeler, P. R., Honore, N., Garnier, T., Churcher, C., Harris, D., Mungall, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R. M., Devlin, K., Duthoy, S., Feltwell, T., Fraser, A., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Lacroix, C., Maclean, J., Moule, S., Murphy, L., Oliver, K., Quail, M. A., Rajandream, M. A., Rutherford, K. M., Rutter, S., Seeger, K., Simon, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Taylor, K., Whitehead, S., Woodward, J. R., & Barrell, B. G. 2001, "Massive gene decay in the leprosy bacillus", *Nature*, vol. 409, no. 6823, pp. 1007-1011.
- Cole, S. T. 1999, "Learning from the genome sequence of *Mycobacterium tuberculosis* H37Rv", *FEBS Letters*, vol. 452, no. 1-2, pp. 7-10.
- Combs, D. L., O'Brien, R. J., & Geiter, L. J. 1990, "USPHS Tuberculosis Short-Course Chemotherapy Trial 21: effectiveness, toxicity, and acceptability. The report of final results", *Ann.Intern.Med.*, vol. 112, no. 6, pp. 397-406.
- Comstock, G. W. 2000, "Epidemiology of tuberculosis," in *Tuberculosis: A comprehensive international approach.*, L. B. Reichmann & E. S. Hershfield, eds., Marcel Dekker, Inc., New York, pp. 129-156.

## References

- Comstock, G. W., Baum, C., & Snider, D. E., Jr. 1979, "Isoniazid prophylaxis among Alaskan Eskimos: a final report of the bethel isoniazid studies", *Am.Rev.Respir.Dis.*, vol. 119, no. 5, pp. 827-830.
- Comstock, G. W. & Woolpert, S. F. 1972, "Preventive treatment of untreated, nonactive tuberculosis in an Eskimo population", *Arch.Environ.Health*, vol. 25, no. 5, pp. 333-337.
- Condra, J. H. & Pauling, C. 1982, "Induction of the SOS system by DNA ligase-deficient growth of *Escherichia coli*", *J.Gen.Microbiol.*, vol. 128, no. 3, pp. 613-621.
- Coninx, R., Mathieu, C., Debacker, M., Mirzoev, F., Ismaelov, A., de Haller, R., & Meddings, D. R. 1999, "First-line tuberculosis therapy and drug-resistant *Mycobacterium tuberculosis* in prisons", *Lancet*, vol. 353, no. 9157, pp. 969-973.
- Cook, T. M., Deitz, W. H., & Goss, W. A. 1966, "Mechanism of action of nalidixic acid on *Escherichia coli*. IV. Effects on the stability of cellular constituents", *The Journal of Bacteriology*, vol. 91, no. 2, pp. 774-779.
- Cooksey, R. C., Morlock, G. P., Glickman, S., & Crawford, J. T. 1997, "Evaluation of a line probe assay kit for characterization of *rpoB* mutations in rifampin-resistant *Mycobacterium tuberculosis* isolates from New York City", *Journal of Clinical Microbiology*, vol. 35, no. 5, pp. 1281-1283.
- Corbett, E. L., Watt, C. J., Walker, N., Maher, D., Williams, B. G., Raviglione, M. C., & Dye, C. 2003, "The Growing Burden of Tuberculosis: Global Trends and Interactions With the HIV Epidemic", *Archives of Internal Medicine*, vol. 163, no. 9, pp. 1009-1021.
- Cox, E. C. 1976, "Bacterial mutator genes and the control of spontaneous mutation", *Annu.Rev.Genet.*, vol. 10, pp. 135-156.
- Crofton, J., Choculet, P., & Maher, D. 1997, *Guidelines for the management of drug-resistant tuberculosis WHO/TB/96-210 (rev 1)*.

## References

- Cullen, M. M., Sam, N. E., Kanduma, E. G., McHugh, T. D., & Gillespie, S. H. 2006, "Direct detection of heteroresistance in *Mycobacterium tuberculosis* using molecular techniques", *Journal of Medical Microbiology*, vol. 55, no. 8, pp. 1157-1158.
- Cundliffe, E. 1981, "Antibiotic inhibitors of ribosome function," in *Molecular basis of antibiotic action*, E. F. Gale et al., eds., John Wiley & Sons Inc., New York, pp. 402-457.
- Dahl, J. L., Kraus, C. N., Boshoff, H. I. M., Doan, B., Foley, K., Avarbock, D., Kaplan, G., Mizrahi, V., Rubin, H., & Barry, C. E., III 2003, "The role of RelMtb-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice", *Proceedings of the National Academy of Sciences*, vol. 100, no. 17, pp. 10026-10031.
- Daniel, T. M., Bates, J. H., & Downes, K. A. 1994, "History of tuberculosis," in *Tuberculosis: Pathogenesis, Protection and Control.*, B. R. Bloom, ed., ASM, Washington, DC, pp. 13-24.
- Dannenberger, A. M. Jr. & Rook, G. A. W. 1994, "Pathogenesis of pulmonary tuberculosis; an interplay between tissue-damaging and macrophage-activating immune responses: dual mechanisms that control bacillary multiplication.," in *Tuberculosis: pathogenesis, protection, and control.*, B. R. Bloom, ed., ASM Press, Washington DC, pp. 459-484.
- David, H. L. & Newman, C. M. 1971, "Some observations on the genetics of isoniazid resistance in the tubercle bacilli", *Am.Rev.Respir.Dis.*, vol. 104, no. 4, pp. 508-515.
- David, S. S. & Williams, S. D. 1998, "Chemistry of Glycosylases and Endonucleases Involved in Base-Excision Repair", *Chem.Rev.*, vol. 98, no. 3, pp. 1221-1262.
- Davies, A. P., Billington, O. J., Bannister, B. A., Weir, W. R., McHugh, T. D., & Gillespie, S. H. 2000a, "Comparison of fitness of two isolates of *Mycobacterium tuberculosis*, one of which had developed multi-drug resistance during the course of treatment", *J.Infect.*, vol. 41, no. 2, pp. 184-187.
- Davies, A. P., Billington, O. J., McHugh, T. D., Mitchison, D. A., & Gillespie, S. H. 2000b, "Comparison of Phenotypic and Genotypic Methods for Pyrazinamide

- Susceptibility Testing with *Mycobacterium tuberculosis*", *Journal of Clinical Microbiology*, vol. 38, no. 10, pp. 3686-3688.
- Davies, P. D. O. 2003, "Respiratory tuberculosis," in *Clinical Tuberculosis*, 3 edn, P. D. O. Davies, ed., Arnold Publishers, London, pp. 107-124.
- Davis, E. O., Dullaghan, E. M., & Rand, L. 2002, "Definition of the Mycobacterial SOS Box and Use To Identify LexA-Regulated Genes in *Mycobacterium tuberculosis*", *The Journal of Bacteriology*, vol. 184, no. 12, pp. 3287-3295.
- Davis, E. O., Springer, B., Gopaul, K. K., Papavinasasundaram, K. G., Sander, P., & Bottger, E. C. 2002, "DNA damage induction of *recA* in *Mycobacterium tuberculosis* independently of RecA and LexA", *Molecular Microbiology*, vol. 46, no. 3, pp. 791-800.
- Dawson, D. 2001, "Tuberculosis in Australia: bacteriologically confirmed cases and drug resistance, 1998-1999: report of the Australian Mycobacterium Reference Laboratory Network", *Commun.Dis.Intell.*, vol. 25, no. 4, pp. 261-265.
- DeMaio, J., Zhang, Y., Ko, C., Young, D. B., & Bishai, W. R. 1996, "A stationary-phase stress-response sigma factor from *Mycobacterium tuberculosis*", *Proceedings of the National Academy of Sciences*, vol. 93, no. 7, pp. 2790-2794.
- Deretic, V., Pagan-Ramos, E., Zhang, Y., Dhandayuthapani, S., & Via, L. E. 1996, "The extreme sensitivity of *Mycobacterium tuberculosis* to the front-line antituberculosis drug isoniazid", *Nat.Biotechnol.*, vol. 14, no. 11, pp. 1557-1561.
- Deretic, V., Philipp, W., Dhandayuthapani, S., Mudd, M. H., Curcic, R., Garbe, T., Heym, B., Via, L. E., & Cole, S. T. 1995, "*Mycobacterium tuberculosis* is a natural mutant with an inactivated oxidative-stress regulatory gene: implications for sensitivity to isoniazid", *Mol.Microbiol.*, vol. 17, no. 5, pp. 889-900.
- Dessen, A., Quemard, A., Blanchard, J. S., Jacobs, W. R., Jr., & Sacchettini, J. C. 1995, "Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*", *Science*, vol. 267, no. 5204, pp. 1638-1641.

Deutsches Zentralkomitee zur Bekämpfung der Tuberkulose 2002, *Informationsbericht*, Main: pmi Verlag, Frankfurt, 27.

Dhandayuthapani, S., Zhang, Y., Mudd, M. H., & Deretic, V. 1996, "Oxidative stress response and its role in sensitivity to isoniazid in mycobacteria: characterization and inducibility of *ahpC* by peroxides in *Mycobacterium smegmatis* and lack of expression in *M. aurum* and *M. tuberculosis*", *Journal of Bacteriology*, vol. 178, no. 12, pp. 3641-3649.

Division of the Tuberculosis Elimination 2001, *Surveillance reports: reported tuberculosis in the United States*, CDC and Prevention, Atlanta.

Dizdaroglu, M., Holwitt, E., Hagan, M. P., & Blakely, W. F. 1986, "Formation of cytosine glycol and 5,6-dihydroxycytosine in deoxyribonucleic acid on treatment with osmium tetroxide", *Biochemical Journal*, vol. 235, no. 2, pp. 531-536.

Dobner, P., Rusch-Gerdes, S., Bretzel, G., Feldmann, K., Rifai, M., Loscher, T., & Rinder, H. 1997, "Usefulness of *Mycobacterium tuberculosis* genomic mutations in the genes *katG* and *inhA* for the prediction of isoniazid resistance", *Int.J.Tuberc.Lung Dis.*, vol. 1, no. 4, pp. 365-369.

Doig, C., Seagar, A. L., Watt, B., & Forbes, K. J. 2002, "The efficacy of the heat killing of *Mycobacterium tuberculosis*", *Journal of Clinical Pathology*, vol. 55, no. 10, pp. 778-779.

Dong, Y., Xu, C., Zhao, X., Domagala, J., & Drlica, K. 1998, "Fluoroquinolone action against mycobacteria: effects of C-8 substituents on growth, survival, and resistance", *Antimicrobial Agents and Chemotherapy*, vol. 42, no. 11, pp. 2978-2984.

Doran, J. L., Pang, Y., Mdluli, K. E., Moran, A. J., Victor, T. C., Stokes, R. W., Mahenthiralingam, E., Kreiswirth, B. N., Butt, J. L., Baron, G. S., Treit, J. D., Kerr, V. J., Van Helden, P. D., Roberts, M. C., & Nano, F. E. 1997, "*Mycobacterium tuberculosis* *efpA* encodes an efflux protein of the QacA transporter family", *Clinical and Vaccine Immunology*, vol. 4, no. 1, pp. 23-32.



- Douglass, J. & Steyn, L. M. 1993, "A ribosomal gene mutation in streptomycin-resistant *Mycobacterium tuberculosis* isolates", *Journal of Infectious Diseases*, vol. 167, no. 6, pp. 1505-1506.
- Doukhan, L., Predich, M., Nair, G., Dussurget, O., Mandic-Mulec, I., Cole, S. T., Smith, D. R., & Smith, I. 1995, "Genomic organization of the mycobacterial sigma gene cluster", *Gene*, vol. 165, pp. 67-70.
- Drake, J. 1970, *The Molecular Basis of Mutation* Holden-Day, San Francisco.
- Draper, P. 1998, "The outer parts of the mycobacterial envelope as permeability barriers", *Front Biosci.*, vol. 3, p. D1253-D1261.
- Dri, A. M. & Moreau, P. L. 1994, "Control of the LexA regulon by pH: evidence for a reversible inactivation of the LexA repressor during the growth cycle of *Escherichia coli*", *Mol. Microbiol.*, vol. 12, no. 4, pp. 621-629.
- Drlica, K. 1999, "Mechanism of fluoroquinolone action", *Curr. Opin. Microbiol.*, vol. 2, no. 5, pp. 504-508.
- Drlica, K. & Hooper, D. C. 2003, "Mechanisms of quinolone action," in *Quinolone Antimicrobial Agents*, 3 edn, D. C. Hooper & E. Rubinstein, eds., ASM Press, Washington DC, pp. 19-40.
- Drlica, K. & Malik, M. 2003, "Fluoroquinolones: Action and Resistance", *Current Topics in Medicinal Chemistry*, vol. 3, pp. 249-282.
- Drlica, K. & Zhao, X. 1997, "DNA gyrase, topoisomerase IV, and the 4-quinolones", *Microbiology and Molecular Biology Reviews*, vol. 61, no. 3, pp. 377-392.
- Drobniewski, F., Eltringham, I., Graham, C., Magee, J. G., Smith, E. G., & Watt, B. 2002, "A national study of clinical and laboratory factors affecting the survival of patients with multiple drug resistant tuberculosis in the UK", *Thorax*, vol. 57, no. 9, pp. 810-816.

Drobniewski, F. A. & Wilson, S. M. 1998, "The rapid diagnosis of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis*--a molecular story", *J.Med.Microbiol.*, vol. 47, no. 3, pp. 189-196.

DSCTAC 2000, "Diagnostic Standards and Classification of Tuberculosis in Adults and Children . This Official Statement of the American Thoracic Society and the Centers for Disease Control and Prevention was Adopted by the ATS Board of Directors, July 1999. This Statement was endorsed by the Council of the Infectious Disease Society of America, September 1999", *American Journal of Respiratory and Critical Care Medicine*, vol. 161, no. 4, pp. 1376-1395.

Dullaghan, E. M., Brooks, P. C., & Davis, E. O. 2002, "The role of multiple SOS boxes upstream of the *Mycobacterium tuberculosis* *lexA* gene - identification of a novel DNA-damage-inducible gene", *Microbiology*, vol. 148, no. 11, pp. 3609-3615.

Dunman, P. M., Ren, L., Rahman, M. S., Palejwala, V. A., Murphy, H. S., Volkert, M. R., & Humayun, M. Z. 2000, "*Escherichia coli* cells defective for the *recN* gene display constitutive elevation of mutagenesis at 3,N(4)-ethenocytosine via an SOS-induced mechanism", *Mol.Microbiol.*, vol. 37, no. 3, pp. 680-686.

Durbach, S. I., Springer, B., Machowski, E. E., North, R. J., Papavinasasundaram, K. G., Colston, M. J., Bottger, E. C., & Mizrahi, V. 2003, "DNA Alkylation Damage as a Sensor of Nitrosative Stress in *Mycobacterium tuberculosis*", *Infection and Immunity*, vol. 71, no. 2, pp. 997-1000.

Dye, C. 2003, "Epidemiology," in *Clinical Tuberculosis*, 3 edn, P. D. O. Davies, ed., Arnold, London, pp. 21-42.

Dye, C. & Espinal, M. A. 2001, "Will tuberculosis become resistant to all antibiotics?", *Proc.R.Soc.Lond B Biol.Sci.*, vol. 268, no. 1462, pp. 45-52.

Dye, C., Espinal, M. A., Watt, C. J., Mbiaga, C., & Williams, B. G. 2002a, "Worldwide incidence of Multi-drug Resistant Tuberculosis", *The Journal of Infectious Diseases*, vol. 185, pp. 1197-1202.

- Dye, C. & Williams, B. G. 2000, "Criteria for the control of drug-resistant tuberculosis", *Proc.Natl.Acad.Sci.U.S.A*, vol. 97, no. 14, pp. 8180-8185.
- Dye, C., Williams, B. G., Espinal, M. A., & Raviglione, M. C. 2002b, "Erasing the World's Slow Strain: Strategies to Beat Multidrug-Resistant Tuberculosis", *Science*, vol. 295, pp. 2042-2046.
- Dye, C., Scheele, S., Dolin, P., Pathania, V., Raviglione, M. C., & for the WHO Global Surveillance and Monitoring Project 1999, "Global Burden of Tuberculosis: Estimated Incidence, Prevalence, and Mortality by Country", *JAMA: The Journal of the American Medical Association*, vol. 282, no. 7, pp. 677-686.
- East African/British Medical Research Council 1981, "Controlled trial of five short course regimens of chemotherapy regimens for pulmonary tuberculosis", *American Review of Respiratory Disease*, vol. 123, pp. 165-170.
- Edwards, F. G. B. 1970, "Disease caused by 'atypical' (opportunistic) mycobacteria: a whole population review.", *Tubercle*, vol. 51, pp. 285-295.
- Eick, S., Schmitt, A., Sachse, S., Schmidt, K. H., & Pfister, W. 2004, "In vitro antibacterial activity of fluoroquinolones against *Porphyromonas gingivalis* strains", *Journal of Antimicrobial Chemotherapy*, vol. 54, no. 2, pp. 553-556.
- Eiglmeier, K., Parkhill, J., Honore, N., Garnier, T., Tekaiia, F., Telenti, A., Klatser, P., James, K. D., Thomson, N. R., Wheeler, P. R., Churcher, C., Harris, D., Mungall, K., Barrell, B. G., & Cole, S. T. 2001, "The decaying genome of *Mycobacterium leprae*", *Lepr.Rev.*, vol. 72, no. 4, pp. 387-398.
- El Baghdadi, J., Remus, N., Laaboudi, L., & Benslimane, A. 2003, "Chronic cases of tuberculosis in Casablanca, Morocco", *International Journal of Tuberculosis and Lung Disease*, vol. 7, no. 7, pp. 660-664.
- Elghoul, M. T., Joshi, R. M., & Rizghalla, T. 1989, "Primary and acquired drug resistance in *Mycobacterium tuberculosis* strains in western region of Libyan Arab Jamahiriya", *Trop.Geogr.Med.*, vol. 41, no. 4, pp. 304-308.

- Enarson, D. A. & Rouillon, A. 1994, "The epidemiological basis of tuberculosis control," in *Clinical Tuberculosis*, P. D. O. Davies, ed., Chapman and Hall, London, pp. 19-32.
- Endoh, T., Yagihashi, A., Uehara, N., Kobayashi, D., Tsuji, N., Nakamura, M., Hayashi, S., Fujii, N., & Watanabe, N. 2002, "Pyrazinamide resistance associated with *pncA* gene mutation in *Mycobacterium tuberculosis* in Japan", *Epidemiology and Infection*, vol. 128, no. 2, pp. 337-342.
- Escalante, P., Ramaswamy, S., Sanabria, H., Soini, H., Pan, X., Valiente-Castillo, O., & Musser, J. M. 1998, "Genotypic characterization of drug-resistant *Mycobacterium tuberculosis* isolates from Peru", *Tubercle and Lung Disease*, vol. 79, no. 2, pp. 111-118.
- Espinal, M. A., Laszlo, A., Simonsen, L., Boulahbal, F., Kim, S. J., Reniero, A., Hoffner, S., Rieder, H. L., Binkin, N., Dye, C., Williams, R., & Ravigliione, M. C. 2001, "Global trends in resistance to antituberculosis drugs. World Health Organization-International Union against Tuberculosis and Lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance", *N.Engl.J.Med.*, vol. 344, no. 17, pp. 1294-1303.
- Fan, X. Y., Hu, Z. Y., Xu, F. H., Yan, Z. Q., Guo, S. Q., & Li, Z. M. 2003, "Rapid detection of *rpoB* gene mutations in rifampin-resistant *Mycobacterium tuberculosis* isolates in shanghai by using the amplification refractory mutation system", *Journal of Clinical Microbiology*, vol. 41, no. 3, pp. 993-997.
- Farmer, P. & Kim, J. Y. 1998, "Community based approaches to the control of multidrug resistant tuberculosis: introducing "DOTS-plus"", *BMJ*, vol. 317, no. 7159, pp. 671-674.
- Fedorov, Y. 1999, *Human Development Report 1999, Russian Federation. United Nations Development Programme.*, Human Rights Publishers, Moscow.
- Ferguson, J. S. & Schlesinger, L. S. 2000, "Pulmonary surfactant in innate immunity and the pathogenesis of tuberculosis", *Tubercle and Lung Disease*, vol. 80, no. 4-5, pp. 173-184.

- Finken, M., Kirschner, P., Meier, A., Wrede, A., & Bottger, E. C. 1993, "Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot", *Molecular Microbiology*, vol. 9, no. 6, pp. 1239-1246.
- Fisher, M. A., Plikaytis, B. B., & Shinnick, T. M. 2002, "Microarray Analysis of the *Mycobacterium tuberculosis* Transcriptional Response to the Acidic Conditions Found in Phagosomes", *The Journal of Bacteriology*, vol. 184, no. 14, pp. 4025-4032.
- Fitzgerald, J. M. & Menzies, D. 2003, "Interpretation of the tuberculin skin test," in *Clinical Tuberculosis*, 3 edn, P. D. O. Davies, ed., Arnold Publishers, London, pp. 323-336.
- Fluit, A. C., Wielders, C. L. C., Verhoef, J., & Schmitz, F. J. 2001, "Epidemiology and Susceptibility of 3,051 *Staphylococcus aureus* Isolates from 25 University Hospitals Participating in the European SENTRY Study", *Journal of Clinical Microbiology*, vol. 39, no. 10, pp. 3727-3732.
- Flynn, J. L. & Chan, J. 2001, "Immunology of tuberculosis", *Annu.Rev.Immunol*, vol. 19, pp. 93-129.
- Fowler, R. G., Degnen, G. E., & Cox, E. C. 1974, "Mutational specificity of a conditional *Escherichia coli* mutator, *mutD5*", *Mol.Gen.Genet.*, vol. 133, no. 3, pp. 179-191.
- Fowler, R. G., White, S. J., Koyama, C., Moore, S. C., Dunn, R. L., & Schaaper, R. M. 2003, "Interactions among the *Escherichia coli* *mutT*, *mutM*, and *mutY* damage prevention pathways", *DNA Repair (Amst)*, vol. 2, no. 2, pp. 159-173.
- Fox, H. H. 1951, "Synthetic Tuberculostatics show promise.", *Chem Eng News*, vol. 29, pp. 3963-3964.
- Fox, W. 1958, "The problem of self-administration of drugs; with particular reference to pulmonary tuberculosis", *Tubercle*, vol. 39, no. 5, pp. 269-274.

Fraser, C. M., Norris, S. J., Weinstock, G. M., White, O., Sutton, G. G., Dodson, R., Gwinn, M., Hickey, E. K., Clayton, R., Ketchum, K. A., Sodergren, E., Hardham, J. M., McLeod, M. P., Salzberg, S., Peterson, J., Khalak, H., Richardson, D., Howell, J. K., Chidambaram, M., Utterback, T., McDonald, L., Artiach, P., Bowman, C., Cotton, M. D., Fujii, C., Garland, S., Hatch, B., Horst, K., Roberts, K., Sandusky, M., Weidman, J., Smith, H. O., & Venter, J. C. 1998, "Complete Genome Sequence of *Treponema pallidum*, the Syphilis Spirochete", *Science*, vol. 281, no. 5375, pp. 375-388.

Friedberg, E., Walker, G. C., & Siede, W. 1995, *DNA Repair and Mutagenesis* American Society for Microbiology Press, Washington D.C.

Frieden, T. R., Fujiwara, P. I., Washko, R. M., & Hamburg, M. A. 1995, "Tuberculosis in New York City -- Turning the Tide", *The New England Journal of Medicine*, vol. 333, no. 4, pp. 229-233.

Fung-Tomc, J., Kolek, B., & Bonner, D. P. 1993, "Ciprofloxacin-induced, low-level resistance to structurally unrelated antibiotics in *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*", *Antimicrobial Agents and Chemotherapy*, vol. 37, no. 6, pp. 1289-1296.

Fung-Tomc, J., Minassian, B., Kolek, B., Washo, T., Huczko, E., & Bonner, D. 2000, "*In vitro* antibacterial spectrum of a new broad-spectrum 8-methoxy fluoroquinolone, gatifloxacin", *Journal of Antimicrobial Chemotherapy*, vol. 45, no. 4, pp. 437-446.

Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H., & Waring, M. J. 1981, *The Molecular Basis of Antibiotic Action* John Wiley & Sons, New York.

Gangadharam, P. R., Perumal, V. K., Jairam, B. T., Rao, P. N., Nguyen, A. K., Farhi, D. C., & Iseman, M. D. 1987, "Activity of rifabutin alone or in combination with clofazimine or ethambutol or both against acute and chronic experimental *Mycobacterium intracellulare* infections", *Am.Rev.Respir.Dis.*, vol. 136, no. 2, pp. 329-333.

- Garcia, d., V, del, S. D., I, Lasala, F., Chaves, F., Alcala, L., & Bouza, E. 2002, "New real-time PCR able to detect in a single tube multiple rifampin resistance mutations and high-level isoniazid resistance mutations in *Mycobacterium tuberculosis*", *Journal of Clinical Microbiology*, vol. 40, no. 3, pp. 988-995.
- Garcia, L., Alonso-Sanz, M., Rebollo, M. J., Tercero, J. C., & Chaves, F. 2001, "Mutations in the *rpoB* gene of rifampin-resistant *Mycobacterium tuberculosis* isolates in Spain and their rapid detection by PCR-enzyme-linked immunosorbent assay", *Journal of Clinical Microbiology*, vol. 39, no. 5, pp. 1813-1818.
- Garg, S. K., Tiwari, R. P., Tiwari, D., Singh, R., Malhotra, D., Ramnani, V. K., Prasad, G. B., Chandra, R., Fraziano, M., Colizzi, V., & Bisen, P. S. 2003, "Diagnosis of tuberculosis: available technologies, limitations, and possibilities", *J Clin Lab Anal.*, vol. 17, no. 5, pp. 155-163.
- Gaudier, B. & Gernez-Rieux, C. 1962, "Étude expérimentale de la vitalité du B.C.G. au cours de la traversée gastro-intestinale chez des enfants non allergiques vaccinés par voie digestive.", *Ann.Inst.Pasteur Lille*, vol. 13, pp. 77-87.
- Gedde-Dahl, T. 1952, "Tuberculosis infection in the light of tuberculin matriculation", *Am.J.Hyg.*, vol. 56, no. 2, pp. 139-214.
- George, K. M., Chatterjee, D., Gunawardana, G., Welty, D., Hayman, J., Lee, R., & Small, P. L. 1999, "Mycolactone: A Polyketide Toxin from *Mycobacterium ulcerans* Required for Virulence", *Science*, vol. 283, no. 5403, pp. 854-857.
- Ghaffar, F. & McCracken, G. H. Jr. 2003, "Quinolones in Pediatrics," in *Quinolone Antimicrobial Agents*, 3 edn, D. C. Hooper & E. Rubinstein, eds., ASM Press, Washington DC, pp. 343-355.
- Gillespie, S. H. 2001, "Antibiotic resistance in the absence of selective pressure", *Int.J.Antimicrob.Agents*, vol. 17, pp. 171-176.
- Gillespie, S. H. & Billington, O. 1999, "Activity of moxifloxacin against mycobacteria", *Journal of Antimicrobial Chemotherapy*, vol. 44, no. 3, pp. 393-395.

- Gillespie, S. H. & Kennedy, N. 1998, "Fluoroquinolones: a new treatment for tuberculosis?", *Int.J.Tuberc.Lung Dis.*, vol. 2, no. 4, pp. 265-271.
- Gillespie, S. H. & McHugh, T. D. 1997, "Monitoring the therapy of pulmonary tuberculosis by nested polymerase chain reaction", *J Infect.*, vol. 35, no. 3, pp. 324-325.
- Gillespie, S. H., Voelker, L. L., Ambler, J. E., Traini, C., & Dickens, A. 2003, "Fluoroquinolone resistance in *Streptococcus pneumoniae*: evidence that *gyrA* mutations arise at a lower rate and that mutation in *gyrA* or *parC* predisposes to further mutation", *Microb.Drug Resist.*, vol. 9, no. 1, pp. 17-24.
- Gillespie, S. H. 2002, "Evolution of Drug Resistance in *Mycobacterium tuberculosis*: Clinical and Molecular Perspective", *Antimicrobial Agents and Chemotherapy*, vol. 46, no. 2, pp. 267-274.
- Gillespie, S. H., Basu, S., Dickens, A. L., O'Sullivan, D. M., & McHugh, T. D. 2005, "Effect of subinhibitory concentrations of ciprofloxacin on *Mycobacterium fortuitum* mutation rates", *Journal of Antimicrobial Chemotherapy*, vol. 56, no. 2, pp. 344-348.
- Gillespie, S. H., Morrissey, I., & Everett, D. 2001, "A comparison of the bactericidal activity of quinolone antibiotics in a *Mycobacterium fortuitum* model", *J.Med Microbiol.*, vol. 50, no. 6, pp. 565-570.
- Gillespie, S. H., Gosling, R. D., & Charalambous, B. M. 2002, "A Reiterative Method for Calculating the Early Bactericidal Activity of Antituberculosis Drugs", *American Journal of Respiratory and Critical Care Medicine*, vol. 166, no. 1, pp. 31-35.
- Gilroy, N., Oliver, G., & Harvey, B. 1998, "Tuberculosis notifications in Australia, 1996. Communicable Diseases Network Australia New Zealand", *Commun.Dis.Intell.*, vol. 22, no. 9, pp. 173-183.
- Ginsburg, A. S., Grosset, J. H., & Bishai, W. R. 2003, "Fluoroquinolones, tuberculosis, and resistance", *Lancet Infect.Dis.*, vol. 3, no. 7, pp. 432-442.



- Girardi, E., Raviglione, M. C., Antonucci, G., Godfrey-Faussett, P., & Ippolito, G. 2000, "Impact of the HIV epidemic on the spread of other diseases: the case of tuberculosis", *AIDS*, vol. 14 Suppl 3, p. S47-S56.
- Glickman, B. W. 1979, "Spontaneous mutagenesis in *Escherichia coli* strains lacking 6-methyladenine residues in their DNA: an altered mutational spectrum in dam- mutants", *Mutation Research*, vol. 61, no. 2, pp. 153-162.
- Glickman, M. S. & Jacobs, J. 2001, "Microbial Pathogenesis of *Mycobacterium tuberculosis*: Dawn of a Discipline", *Cell*, vol. 104, no. 4, pp. 477-485.
- Glickman, S. E., Kilburn, J. O., Butler, W. R., & Ramos, L. S. 1994, "Rapid identification of mycolic acid patterns of mycobacteria by high-performance liquid chromatography using pattern recognition software and a Mycobacterium library", *Journal of Clinical Microbiology*, vol. 32, no. 3, pp. 740-745.
- Goerlich, O., Quillardet, P., & Hofnung, M. 1989, "Induction of the SOS response by hydrogen peroxide in various *Escherichia coli* mutants with altered protection against oxidative DNA damage", *The Journal of Bacteriology*, vol. 171, no. 11, pp. 6141-6147.
- Goh, E. B., Yim, G., Tsui, W., McClure, J., Surette, M. G., & Davies, J. 2002, "Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics", *Proceedings of the National Academy of Sciences*, vol. 99, no. 26, pp. 17025-17030.
- Gonzalez, N., Torres, M. J., Aznar, J., & Palomares, J. C. 1999, "Molecular analysis of rifampin and isoniazid resistance of *Mycobacterium tuberculosis* clinical isolates in Seville, Spain", *Tubercle and Lung Disease*, vol. 79, no. 3, pp. 187-190.
- Goodman, M. F. 2002, "Error-prone repair DNA polymerases in prokaryotes and eukaryotes", *Annual Review of Biochemistry*, vol. 71, no. 1, pp. 17-50.
- Gordon, A. H. & Hart, P. D. 1994, "Stimulation or inhibition of the respiratory burst in cultured macrophages in a mycobacterium model: initial stimulation is followed by inhibition after phagocytosis", *Infection and Immunity*, vol. 62, no. 10, pp. 4650-4651.

## References

- Goren, M. B. & Brennan, P. J. 1980, "Mycobacterial lipids: chemistry and biological activities.," in *Tuberculosis*, G. P. Youmans, ed., W.B. Saunders, Philadelphia, pp. 63-193.
- Gosling, R. D., Uiso, L. O., Sam, N. E., Bongard, E., Kanduma, E. G., Nyindo, M., Morris, R. W., & Gillespie, S. H. 2003, "The Bactericidal Activity of Moxifloxacin in Patients with Pulmonary Tuberculosis", *American Journal of Respiratory and Critical Care Medicine*, vol. 168, no. 11, pp. 1342-1345.
- Gradelski, E., Valera, L., Bonner, D., & Fung-Tomc, J. 2001, "Synergetic activities of gatifloxacin in combination with other antimicrobial agents against *Pseudomonas aeruginosa* and related species", *Antimicrobial Agents and Chemotherapy*, vol. 45, pp. 3220-3222.
- Gradelski, E., Kolek, B., Bonner, D., & Fung-Tomc, J. 2002, "Bactericidal mechanism of gatifloxacin compared with other quinolones", *Journal of Antimicrobial Chemotherapy*, vol. 49, no. 1, pp. 185-188.
- Grange, J. M. 2003, "Immunophysiology and immunopathology," in *Clinical Tuberculosis*, 3 edn, P. D. O. Davies, ed., Arnold, London, pp. 88-104.
- Greene, R. 1992, "Fleischner Lecture. Imaging the respiratory system in the first few years after discovery of the X-ray: contributions of Francis H. Williams, M.D", *AJR Am.J.Roentgenol.*, vol. 159, no. 1, pp. 1-7.
- Grollman, A. P. & Moriya, M. 1993, "Mutagenesis by 8-oxoguanine: an enemy within", *Trends in Genetics*, vol. 9, no. 7, pp. 246-249.
- Grosset, J. 1978, "The sterilizing value of rifampicin and pyrazinamide in experimental short-course chemotherapy", *Bull.Int.Union Tuberc.*, vol. 53, pp. 5-12.
- Grosset, J. 1993, in *Tuberculosis: A comprehensive international approach.*, L. B. Reichmann & E. S. Hershfield, eds., Marcel Dekker, New York, pp. 49-74.

- Gudas, L. J. & Pardee, A. B. 1975, "Model for regulation of *Escherichia coli* DNA repair functions", *Proc.Natl.Acad.Sci.U.S.A*, vol. 72, no. 6, pp. 2330-2334.
- Gudas, L. J. & Pardee, A. B. 1976, "DNA synthesis inhibition and the induction of protein X in *Escherichia coli*", *J.Mol.Biol.*, vol. 101, no. 4, pp. 459-477.
- Guerrero, R., Llagostera, M., Villaverde, A., & Barbe, J. 1984, "Changes in ATP concentration in *Escherichia coli* during induction of the SOS system by mitomycin C and bleomycin", *J.Gen.Microbiol.*, vol. 130, no. 9, pp. 2247-2251.
- Guillemin, I., Cambau, E., & Jarlier, V. 1995, "Sequences of conserved region in the A subunit of DNA gyrase from nine species of the genus *Mycobacterium*: phylogenetic analysis and implication for intrinsic susceptibility to quinolones", *Antimicrobial Agents and Chemotherapy*, vol. 39, no. 9, pp. 2145-2149.
- Guillemin, I., Jarlier, V., & Cambau, E. 1998, "Correlation between quinolone susceptibility patterns and sequences in the A and B subunits of DNA gyrase in mycobacteria", *Antimicrobial Agents and Chemotherapy*, vol. 42, no. 8, pp. 2084-2088.
- Guimaraes, B. G., Souchon, H., Honore, N., Saint-Joanis, B., Brosch, R., Shepard, W., Cole, S. T., & Alzari, P. M. 2005, "Structure and Mechanism of the Alkyl Hydroperoxidase AhpC, a Key Element of the *Mycobacterium tuberculosis* Defense System against Oxidative Stress", *Journal of Biological Chemistry*, vol. 280, no. 27, pp. 25735-25742.
- Gupta, R., Kim, J. Y., Espinal, M. A., Caudron, J. M., Pecoul, B., Farmer, P. E., & Raviglione, M. C. 2001, "PUBLIC HEALTH: Responding to Market Failures in Tuberculosis Control", *Science*, vol. 293, no. 5532, pp. 1049-1051.
- Gutmann, L., Williamson, R., Moreau, N., Kitzis, M. D., Collatz, E., Acar, J. F., & Goldstein, F. W. 1985, "Cross-resistance to nalidixic acid, trimethoprim, and chloramphenicol associated with alterations in outer membrane proteins of *Klebsiella*, *Enterobacter*, and *Serratia*", *J.Infect.Dis.*, vol. 151, no. 3, pp. 501-507.

- Haas, F. & Haas, S. S. 1996, "The origins of *Mycobacterium tuberculosis* and the notion of its contagiousness.," in *Tuberculosis*, W. N. Rom & S. Garay, eds., Little, Brown and Company, Boston, pp. 4-19.
- Haas, W. H., Schilke, K., Brand, J., Amthor, B., Weyer, K., Fourie, P. B., Bretzel, G., Groh, V., & Bremer, H. J. 1997, "Molecular analysis of *katG* gene mutations in strains of *Mycobacterium tuberculosis* complex from Africa", *Antimicrobial Agents and Chemotherapy*, vol. 41, no. 7, pp. 1601-1603.
- Hallett, P. & Maxwell, A. 1991, "Novel quinolone resistance mutations of the *Escherichia coli* DNA gyrase A protein: enzymatic analysis of the mutant proteins", *Antimicrobial Agents and Chemotherapy*, vol. 35, no. 2, pp. 335-340.
- Handa, P., Acharya, N., & Varshney, U. 2001, "Chimeras between Single-stranded DNA-binding Proteins from *Escherichia coli* and *Mycobacterium tuberculosis* Reveal That Their C-terminal Domains Interact with Uracil DNA Glycosylases", *Journal of Biological Chemistry*, vol. 276, no. 20, pp. 16992-16997.
- Hannan, M. M., Desmond, E. P., Morlock, G. P., Mazurek, G. H., & Crawford, J. T. 2001, "Pyrazinamide-monoresistant *Mycobacterium tuberculosis* in the United States", *Journal of Clinical Microbiology*, vol. 39, no. 2, pp. 647-650.
- Harris, K. A., Jr., Mukundan, U., Musser, J. M., Kreiswirth, B. N., & Lalitha, M. K. 2000, "Genetic diversity and evidence for acquired antimicrobial resistance in *Mycobacterium tuberculosis* at a large hospital in South India", *International Journal of Infectious Diseases*, vol. 4, no. 3, pp. 140-147.
- Hastings, R. C. 1994, *Leprosy*, 2 edn, Churchill Livingstone, Edinburgh.
- Heep, M., Brandstatter, B., Rieger, U., Lehn, N., Richter, E., Rusch-Gerdes, S., & Niemann, S. 2001, "Frequency of *rpoB* mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates", *Journal of Clinical Microbiology*, vol. 39, no. 1, pp. 107-110.

- Heep, M., Odenbreit, S., Beck, D., Decker, J., Prohaska, E., Rieger, U., & Lehn, N. 2000, "Mutations at four distinct regions of the *rpoB* gene can reduce the susceptibility of *Helicobacter pylori* to rifamycins", *Antimicrobial Agents and Chemotherapy*, vol. 44, no. 6, pp. 1713-1715.
- Heep, M., Rieger, U., Beck, D., & Lehn, N. 2000, "Mutations in the Beginning of the *rpoB* Gene Can Induce Resistance to Rifamycins in both *Helicobacter pylori* and *Mycobacterium tuberculosis*", *Antimicrobial Agents and Chemotherapy*, vol. 44, no. 4, pp. 1075-1077.
- Heifets, L. & Lindholm-Levy, P. 1992, "Pyrazinamide sterilizing activity *in vitro* against semidormant *Mycobacterium tuberculosis* bacterial populations", *Am.Rev.Respir.Dis.*, vol. 145, no. 5, pp. 1223-1225.
- Heifets, L. B. 1991, "Drug susceptibility tests in the management of chemotherapy of tuberculosis," in *Drug susceptibility in the chemotherapy of mycobacterial infections.*, L. B. Heifets, ed., Boca Raton, CRC Press, Florida, pp. 89-122.
- Hemsworth, G. R. & Kochan, I. 1978, "Secretion of antimycobacterial fatty acids by normal and activated macrophages", *Infection and Immunity*, vol. 19, no. 1, pp. 170-177.
- Hengge-Aronis, R. 2002, "Signal Transduction and Regulatory Mechanisms Involved in Control of the  $\sigma^S$  (RpoS) Subunit of RNA Polymerase", *Microbiology and Molecular Biology Reviews*, vol. 66, no. 3, pp. 373-395.
- Herbert, D., Paramasivan, C. N., Venkatesan, P., Kubendiran, G., Prabhakar, R., & Mitchison, D. A. 1996, "Bactericidal action of ofloxacin, sulbactam-ampicillin, rifampin, and isoniazid on logarithmic- and stationary-phase cultures of *Mycobacterium tuberculosis*", *Antimicrobial Agents and Chemotherapy*, vol. 40, no. 10, pp. 2296-2299.
- Hermans, P. W., Van Soolingen, D., Dale, J. W., Schuitema, A. R., McAdam, R. A., Catty, D., & van Embden, J. D. 1990, "Insertion element IS986 from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis", *Journal of Clinical Microbiology*, vol. 28, no. 9, pp. 2051-2058.

## References

- Hernandez-Pando, R., Aguilar, D., Orozco, H., Jeyanathan, M., Rook, G. A. W., Mengistu, G., Harboe, M., Harboe, M., & Bjune, G. 2000, "Persistence of DNA from *Mycobacterium tuberculosis* in superficially normal lung tissue during latent infection", *The Lancet*, vol. 356, no. 9248, pp. 2133-2138.
- Heym, B., Alzari, P. M., Honore, N., & Cole, S. T. 1995, "Missense mutations in the catalase-peroxidase gene, *katG*, are associated with isoniazid resistance in *Mycobacterium tuberculosis*", *Mol.Microbiol.*, vol. 15, no. 2, pp. 235-245.
- Heym, B. & Cole, S. T. 1992, "Isolation and characterization of isoniazid-resistant mutants of *Mycobacterium smegmatis* and *M. aurum*", *Res.Microbiol.*, vol. 143, no. 7, pp. 721-730.
- Heym, B., Honore, N., Truffot-Pernot, C., Banerjee, A., Schurra, C., Jacobs, W. R., Jr., van Embden, J. D., Grosset, J. H., & Cole, S. T. 1994, "Implications of multidrug resistance for the future of short-course chemotherapy of tuberculosis: a molecular study", *Lancet*, vol. 344, no. 8918, pp. 293-298.
- Hiramatsu, K., Aritaka, N., Hanaki, H., Kawasaki, S., Hosoda, Y., Hori, S., Fukuchi, Y., & Kobayashi, I. 1997, "Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin", *The Lancet*, vol. 350, no. 9092, pp. 1670-1673.
- Hirano, K., Abe, C., & Takahashi, M. 1999, "Mutations in the *rpoB* gene of rifampin-resistant *Mycobacterium tuberculosis* strains isolated mostly in Asian countries and their rapid detection by line probe assay", *Journal of Clinical Microbiology*, vol. 37, no. 8, pp. 2663-2666.
- Hiriyanna, K. T. & Ramakrishnan, T. 1986, "Deoxyribonucleic acid replication time in *Mycobacterium tuberculosis* H37 Rv", *Arch.Microbiol.*, vol. 144, no. 2, pp. 105-109.
- Hoffmann, G. R., Crowley, D. J., & Theophiles, P. J. 2002, "Comparative potencies of induction of point mutations and genetic duplications by the methylating agents

methylazoxymethanol and dimethyl sulfate in bacteria", *Mutagenesis*, vol. 17, no. 5, pp. 439-444.

Holland, S. M. 2001, "Nontuberculous mycobacteria", *Am.J.Med.Sci.*, vol. 321, no. 1, pp. 49-55.

Honer Zu Bentrup, K., Miczak, A., Swenson, D., & Russell, D. G. 1999, "Characterization of activity and expression of isocitrate lyase in *Mycobacterium avium* and *Mycobacterium tuberculosis*", *The Journal of Bacteriology*, vol. 181, pp. 7161-7167.

Honore, N. & Cole, S. T. 1994, "Streptomycin resistance in mycobacteria", *Antimicrobial Agents and Chemotherapy*, vol. 38, no. 2, pp. 238-242.

Hooper, D. C. & Wolfson, J. S. 1985, "The fluoroquinolones: pharmacology, clinical uses, and toxicities in humans", *Antimicrobial Agents and Chemotherapy*, vol. 28, no. 5, pp. 716-721.

Hooper, D. C., Wolfson, J. S., Souza, K. S., Tung, C., McHugh, G. L., & Swartz, M. N. 1986, "Genetic and biochemical characterization of norfloxacin resistance in *Escherichia coli*", *Antimicrobial Agents and Chemotherapy*, vol. 29, no. 4, pp. 639-644.

Hooper, D. C. 2002, "Fluoroquinolone resistance among Gram-positive cocci", *The Lancet Infectious Diseases*, vol. 2, no. 9, pp. 530-538.

Hopewell, P. C. 1994, "Overview of clinical tuberculosis," in *Tuberculosis: Pathogenesis, protection and control.*, B. R. Bloom, ed., ASM Press, Washington DC, pp. 25-46.

Horswill, A. R. & Escalante-Semerena, J. C. 2001, "*In vitro* conversion of propionate to pyruvate by *Salmonella enterica* enzymes: 2-methylcitrate dehydratase (prpd) and aconitase enzymes catalyze the conversion of 2-methylcitrate to 2-methylisocitrate", *Biochemistry*, vol. 40, pp. 4703-4713.

Houston, S. 1998, "Tuberculosis in refugees and displaced persons", *Int.J.Tuberc.Lung Dis*, vol. 2, no. 9 Suppl 1, p. S94-S97.

- Howard, B. M., Pinney, R. J., & Smith, J. T. 1993, "Function of the SOS process in repair of DNA damage induced by modern 4-quinolones", *J.Pharm.Pharmacol.*, vol. 45, no. 7, pp. 658-662.
- Hu, Y. & Coates, A. R. 1999, "Transcription of Two Sigma 70 Homologue Genes, *sigA* and *sigB*, in Stationary-Phase *Mycobacterium tuberculosis*", *The Journal of Bacteriology*, vol. 181, no. 2, pp. 469-476.
- Hu, Y. & Coates, A. R. M. 2003, "Tuberculosis," in *Dormancy and Low-Growth States in Microbial Disease*, A. R. M. Coates, ed., Cambridge, pp. 181-202.
- Hu, Y., Butcher, P. D., Mangan, J. A., Rajandream, M. A., & Coates, A. R. 1999, "Regulation of *hmp* Gene Transcription in *Mycobacterium tuberculosis*: Effects of Oxygen Limitation and Nitrosative and Oxidative Stress", *The Journal of Bacteriology*, vol. 181, no. 11, pp. 3486-3493.
- Hu, Y., Kendall, S., Stoker, N. G., & Coates, A. R. 2004, "The *Mycobacterium tuberculosis sigJ* gene controls sensitivity of the bacterium to hydrogen peroxide", *FEMS Microbiol.Lett.*, vol. 237, no. 2, pp. 415-423.
- Huang, J., Hsu, D. S., Kazantsev, A., & Sancar, A. 1994, "Substrate Spectrum of Human Excinuclease: Repair of Abasic Sites, Methylated Bases, Mismatches, and Bulky Adducts", *Proceedings of the National Academy of Sciences*, vol. 91, no. 25, pp. 12213-12217.
- Huberts, P. & Mizrahi, V. 1995, "Cloning and sequence analysis of the gene encoding the DNA polymerase I from *Mycobacterium tuberculosis*", *Gene*, vol. 164, no. 1, pp. 133-136.
- Huggett, J. F., McHugh, T. D., & Zumla, A. 2003, "Tuberculosis: amplification-based clinical diagnostic techniques", *The International Journal of Biochemistry & Cell Biology*, vol. 35, no. 10, pp. 1407-1412.



- Hwang, H. Y., Chang, C. Y., Chang, L. L., Chang, S. F., Chang, Y. H., & Chen, Y. J. 2003, "Characterization of rifampicin-resistant *Mycobacterium tuberculosis* in Taiwan", *Journal of Medical Microbiology*, vol. 52, no. Pt 3, pp. 239-245.
- Imaeda, T. 1985, "Deoxyribonucleic acid relatedness among selected strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium micoti* and *Mycobacterium africanum*.", *Int.J.Syst.Bacteriol.*, vol. 35, pp. 147-150.
- Irish, C., Herbert, J., Bennett, D., Gilham, C., Drobniowski, F., Williams, R., Smith, E. G., Magee, J. G., Watt, B., Chadwick, M., & Watson, J. M. 1999, "Database study of antibiotic resistant tuberculosis in the United Kingdom, 1994-6", *BMJ*, vol. 318, no. 7182, pp. 497-498.
- Iseman, M. D. 1994, "Evolution of Drug-Resistant Tuberculosis: A Tale of Two Species", *Proceedings of the National Academy of Sciences*, vol. 91, no. 7, pp. 2428-2429.
- Iseman, M. D., Cohn, D. L., & Sbarbaro, J. A. 1993, "Directly Observed Treatment of Tuberculosis -- We Can't Afford Not to Try It", *The New England Journal of Medicine*, vol. 328, no. 8, pp. 576-578.
- Ito, T., Matsumoto, M., & Nishino, T. 1995, "Improved bactericidal activity of Q-35 against quinolone-resistant staphylococci", *Antimicrobial Agents and Chemotherapy*, vol. 39, no. 7, pp. 1522-1525.
- Jaber, M., Rattan, A., & Kumar, R. 1996, "Presence of *katG* gene in resistant *Mycobacterium tuberculosis*", *J.Clin.Pathol.*, vol. 49, no. 11, pp. 945-947.
- Jackett, P. S., Aber, V. R., & Lowrie, D. B. 1978, "Virulence and resistance to superoxide, low pH and hydrogen peroxide among strains of *Mycobacterium tuberculosis*", *Journal of General Microbiology*, vol. 104, no. 1, pp. 37-45.
- Jacobson, F. S., Morgan, R. W., Christman, M. F., & Ames, B. N. 1989, "An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA

against oxidative damage. Purification and properties", *Journal of Biological Chemistry*, vol. 264, no. 3, pp. 1488-1496.

Jenkins, C., Claxton, A. P., Shorten, R. J., McHugh, T. D., & Gillespie, S. H. 2005, "Rifampicin resistance in tuberculosis outbreak, London, England", *Emerging Infectious Diseases*, vol. 11, no. 6, pp. 931-934.

Jensen-Cain, D. M. & Quinn, F. D. 2001, "Differential expression of *sigE* by *Mycobacterium tuberculosis* during intracellular growth", *Microb.Pathog.*, vol. 30, no. 5, pp. 271-278.

Ji, B., Lounis, N., Maslo, C., Truffot-Pernot, C., Bonnafoos, P., & Grosset, J. 1998, "*In vitro* and *in vivo* activities of moxifloxacin and clinafloxacin against *Mycobacterium tuberculosis*", *Antimicrobial Agents and Chemotherapy*, vol. 42, no. 8, pp. 2066-2069.

Ji, B., Lounis, N., Truffot-Pernot, C., & Grosset, J. 1995, "*In vitro* and *in vivo* activities of levofloxacin against *Mycobacterium tuberculosis*", *Antimicrobial Agents and Chemotherapy*, vol. 39, no. 6, pp. 1341-1344.

Ji, B., Truffot-Pernot, C., Lacroix, C., Raviglione, M. C., O'Brien, R. J., Olliaro, P., Roscigno, G., & Grosset, J. 1993, "Effectiveness of rifampin, rifabutin, and rifapentine for preventive therapy of tuberculosis in mice", *Am.Rev.Respir.Dis.*, vol. 148, no. 6 Pt 1, pp. 1541-1546.

Jindani, A., Aber, V. R., Edwards, E. A., & Mitchison, D. A. 1980, "The early bactericidal activity of drugs in patients with pulmonary tuberculosis", *Am.Rev.Respir.Dis.*, vol. 121, no. 6, pp. 939-949.

Johnsson, K. & Schultz, P. G. 1994, "Mechanistic Studies of the Oxidation of Isoniazid by the Catalase Peroxidase from *Mycobacterium tuberculosis*", *Journal of The American Chemical Society*, vol. 116, no. 16, pp. 7425-7426.

Joint Tuberculosis Committee of the British Thoracic Society 1990, "Chemotherapy and management of tuberculosis in the United Kingdom: recommendations of the Joint Tuberculosis Committee of the British Thoracic Society.", *Thorax*, vol. 45, pp. 403-408.

- Joint Tuberculosis Committee of the British Thoracic Society 1998, "Chemotherapy and management of tuberculosis in the United Kingdom: recommendations 1998", *Thorax*, vol. 53, no. 7, pp. 536-548.
- Jones, M. E., Thomas, S. M., & Rogers, A. 1994, "Luria-Delbruck fluctuation experiments: design and analysis", *Genetics*, vol. 136, no. 3, pp. 1209-1216.
- Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., Van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., & van Embden, J. 1997, "Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology", *Journal of Clinical Microbiology*, vol. 35, no. 4, pp. 907-914.
- Kanduma, E., McHugh, T. D., & Gillespie, S. H. 2003, "Molecular methods for *Mycobacterium tuberculosis* strain typing: a users guide", *Journal of Applied Microbiology*, vol. 94, no. 5, pp. 781-791.
- Kapur, V., Li, L. L., Hamrick, M. R., Plikaytis, B. B., Shinnick, T. M., Telenti, A., Jacobs, W. R., Jr., Banerjee, A., Cole, S., Yuen, K. Y., & . 1995, "Rapid *Mycobacterium* species assignment and unambiguous identification of mutations associated with antimicrobial resistance in *Mycobacterium tuberculosis* by automated DNA sequencing", *Arch.Pathol.Lab Med.*, vol. 119, no. 2, pp. 131-138.
- Karunakaran, P. & Davies, J. 2000, "Genetic antagonism and hypermutability in *Mycobacterium smegmatis*", *The Journal of Bacteriology*, vol. 182, pp. 3331-3335.
- Karonga Prevention Trial Group 1996, "Randomised controlled trial of single BCG, repeated BCG, or combined BCG and killed *Mycobacterium leprae* vaccine for prevention of leprosy and tuberculosis in Malawi.", *Lancet*, vol. 348, pp. 17-24.
- Karu, A. E. & Belk, E. D. 1982, "Induction of *E. coli* RecA protein via recBC and alternate pathways: quantitation by enzyme-linked immunosorbent assay (ELISA)", *Mol.Gen.Genet.*, vol. 185, no. 2, pp. 275-282.

- Karunakaran, P. & Davies, J. 2000, "Genetic antagonism and hypermutability in *Mycobacterium smegmatis*", *The Journal of Bacteriology*, vol. 182, pp. 3331-3335.
- Kato-Maeda, M., Rhee, J. T., Gingeras, T. R., Salamon, H., Drenkow, J., Smittipat, N., & Small, P. M. 2001, "Comparing Genomes within the Species *Mycobacterium tuberculosis*", *Genome Research*, vol. 11, no. 4, pp. 547-554.
- Keller, C., Lauber, J., Blumenthal, A., Buer, J., & Ehlers, S. 2004, "Resistance and susceptibility to tuberculosis analysed at the transcriptome level: lessons from mouse macrophages", *Tuberculosis.(Edinb.)*, vol. 84, no. 3-4, pp. 144-158.
- Kendall, S. L., Movahedzadeh, F., Rison, S. C. G., Wernisch, L., Parish, T., Duncan, K., Betts, J. C., & Stoker, N. G. 2004, "The *Mycobacterium tuberculosis dosRS* two-component system is induced by multiple stresses", *Tuberculosis*, vol. 84, pp. 247-255.
- Khodursky, A. B., Zechiedrich, E. L., & Cozzarelli, N. R. 1995, "Topoisomerase IV is a Target of Quinolones in *Escherichia coli*", *Proceedings of the National Academy of Sciences*, vol. 92, no. 25, pp. 11801-11805.
- Khomenko, A. G. 1987, "The variability of *Mycobacterium tuberculosis* in patients with cavitary pulmonary tuberculosis in the course of chemotherapy", *Tubercle.*, vol. 68, no. 4, pp. 243-253.
- Kilburn, J. O., Takayama, K., Armstrong, E. L., & Greenberg, J. 1981, "Effects of ethambutol on phospholipid metabolism in *Mycobacterium smegmatis*", *Antimicrobial Agents and Chemotherapy*, vol. 19, no. 2, pp. 346-348.
- Kim, B. J., Lee, S. H., Lyu, M. A., Kim, S. J., Bai, G. H., Chae, G. T., Kim, E. C., Cha, C. Y., & Kook, Y. H. 1999, "Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*)", *J.Clin.Microbiol.*, vol. 37, no. 6, pp. 1714-1720.
- Kim, B. J., Lee, K. H., Park, B. N., Kim, S. J., Park, E. M., Park, Y. G., Bai, G. H., Kim, S. J., & Kook, Y. H. 2001, "Detection of rifampin-resistant *Mycobacterium tuberculosis*

in sputa by nested PCR-linked single-strand conformation polymorphism and DNA sequencing", *Journal of Clinical Microbiology*, vol. 39, no. 7, pp. 2610-2617.

Kim, J. J., Kim, J. G., & Kwon, D. H. 2003, "Mixed-Infection of Antibiotic Susceptible and Resistant *Helicobacter pylori* Isolates in a Single Patient and Underestimation of Antimicrobial Susceptibility Testing", *Helicobacter*, vol. 8, no. 3, pp. 202-206.

Kocagoz, T., Hackbarth, C. J., Unsal, I., Rosenberg, E. Y., Nikaido, H., & Chambers, H. F. 1996, "Gyrase mutations in laboratory-selected, fluoroquinolone-resistant mutants of *Mycobacterium tuberculosis* H37Ra", *Antimicrobial Agents and Chemotherapy*, vol. 40, no. 8, pp. 1768-1774.

Koch, A. L. 1982, "Mutation and growth rates from Luria-Delbrück fluctuation tests", *Mutation Research*, vol. 95, pp. 129-143.

Koch, R. 1882, "Die Aetiologie der Tuberculose.", *Berliner Klinische Wochenschrift*, vol. 19, pp. 221-230.

Koch, R. 1890, "An address on bacteriological research.", *Br.Med.J.*, vol. 2, pp. 380-383.

Koch, W. H. & Woodgate, R. 1998, "The SOS Response in *DNA Damage and Repair* vol 1," in *DNA Repair in Prokaryotes and Lower Eukaryotes*, J. A. Nickoloff & M. F. Hoekstra, eds., Humana Press Inc., Totova, pp. 107-134.

Komp Lindgren, P., Karlsson, A., & Hughes, D. 2003, "Mutation Rate and Evolution of Fluoroquinolone Resistance in *Escherichia coli* Isolates from Patients with Urinary Tract Infections", *Antimicrobial Agents and Chemotherapy*, vol. 47, no. 10, pp. 3222-3232.

Konno, K., Feldmann, F. M., & McDermott, W. 1967, "Pyrazinamide susceptibility and amidase activity of tubercle bacilli", *Am.Rev.Respir.Dis.*, vol. 95, no. 3, pp. 461-469.

Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D., & Rehauer, W. M. 1994, "Biochemistry of homologous recombination in *Escherichia coli*", *Microbiology and Molecular Biology Reviews*, vol. 58, no. 3, pp. 401-465.

- Kreutzer, D. A. & Essigmann, J. M. 1998, "Oxidized, deaminated cytosines are a source of C-->T transitions *in vivo*", *Proceedings of the National Academy of Sciences*, vol. 95, no. 7, pp. 3578-3582.
- Kumar, D., Watson, J. M., Charlett, A., Nicholas, S., & Darbyshire, J. H. 1997, "Tuberculosis in England and Wales in 1993: results of a national survey. Public Health Laboratory Service/British Thoracic Society/Department of Health Collaborative Group", *Thorax*, vol. 52, no. 12, pp. 1060-1067.
- Kurth, R. & Haas, W. H. 2002, "Epidemiology, diagnostic possibilities, and treatment of tuberculosis", *Annals of the Rheumatic Diseases*, vol. 61, no. 90002, pp. 59ii-61.
- Lari, N., Rindi, L., Bonanni, D., Tortoli, E., & Garzelli, C. 2006, "Mutations in *mutT* genes of *Mycobacterium tuberculosis* isolates of Beijing genotype", *Journal of Medical Microbiology*, vol. 55, no. 5, pp. 599-603.
- Layton, J. C. & Foster, P. L. 2003, "Error-prone DNA polymerase IV is controlled by the stress-resonse sigma factor, *RpoS*, in *Escherichia coli*", *Mol.Microbiol.*, vol. 50, pp. 549-561.
- Lea, D. & Coulson, C. 1949, "The distribution of the numbers of mutants in bacterial populations", *J.Genetics*, vol. 49, pp. 264-285.
- Lee, A. S. G., Lim, I. H. K., Tang, L. L. H., Telenti, A., & Wong, S. Y. 1999, "Contribution of *kasA* Analysis to Detection of Isoniazid-Resistant *Mycobacterium tuberculosis* in Singapore", *Antimicrobial Agents and Chemotherapy*, vol. 43, no. 8, pp. 2087-2089.
- Lee, A. S. G., Tang, L. L. H., Lim, I. H. K., & Yew Wong, S. 2002, "Characterization of pyrazinamide and ofloxacin resistance among drug-resistant *Mycobacterium tuberculosis* isolates from Singapore", *International Journal of Infectious Diseases*, vol. 6, no. 1, pp. 48-51.

## References

- Lee, A. S. G., Teo, A. S. M., & Wong, S. Y. 2001, "Novel Mutations in *ndh* in Isoniazid-Resistant *Mycobacterium tuberculosis* Isolates", *Antimicrobial Agents and Chemotherapy*, vol. 45, no. 7, pp. 2157-2159.
- Lee, K. W., Lee, J. M., & Jung, K. S. 2001, "Characterization of *pncA* mutations of pyrazinamide-resistant *Mycobacterium tuberculosis* in Korea", *Journal of Korean Medical Science*, vol. 16, no. 5, pp. 537-543.
- Lenski, R. E. 1998, "Bacterial evolution and the cost of antibiotic resistance", *Int.Microbiol.*, vol. 1, no. 4, pp. 265-270.
- Levin, D. E., Marnett, L. J., & Ames, B. N. 1984, "Spontaneous and Mutagen-Induced Deletions: Mechanistic Studies in Salmonella Tester Strain TA102", *Proceedings of the National Academy of Sciences*, vol. 81, no. 14, pp. 4457-4461.
- Levin, M. E. & Hatfull, G. F. 1993, "*Mycobacterium smegmatis* RNA polymerase: DNA supercoiling, action of rifampicin and mechanism of rifampicin resistance", *Mol.Microbiol.*, vol. 8, no. 2, pp. 277-285.
- Levy-Frebault, V. V. & Portaels, F. 1992, "Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species", *Int.J.Syst.Bacteriol.*, vol. 42, no. 2, pp. 315-323.
- Lewin, C. S., Amyes, S. G., & Smith, J. T. 1989, "Bactericidal activity of enoxacin and lomefloxacin against *Escherichia coli* KL16", *Eur.J.Clin.Microbiol.Infect.Dis.*, vol. 8, no. 8, pp. 731-733.
- Lewin, C. S., Morrissey, I., & Smith, J. T. 1991, "The mode of action of quinolones: the paradox in activity of low and high concentrations and activity in the anaerobic environment", *Eur.J.Clin.Microbiol.Infect.Dis.*, vol. 10, no. 4, pp. 240-248.
- Lillebaek, T., Andersen, A. B., Dirksen, A., Smith, E., Skovgaard, L. T., & Kok-Jensen, A. 2002, "Persistent high incidence of tuberculosis in immigrants in a low-incidence country", *Emerg.Infect.Dis.*, vol. 8, no. 7, pp. 679-684.

- Lillebaek, T., Andersen, A. B., Bauer, J., Dirksen, A., Glismann, S., de Haas, P., & Kok-Jensen, A. 2001, "Risk of *Mycobacterium tuberculosis* Transmission in a Low-Incidence Country Due to Immigration from High-Incidence Areas", *Journal of Clinical Microbiology*, vol. 39, no. 3, pp. 855-861.
- Lindahl, T. 1993, "Instability and decay of the primary structure of DNA", *Nature*, vol. 362, no. 6422, pp. 709-715.
- Lipsitch, M. & Levin, B. R. 1998, "Population dynamics of tuberculosis treatment: mathematical models of the roles of non-compliance and bacterial heterogeneity in the evolution of drug resistance", *Int.J.Tuberc.Lung Dis.*, vol. 2, no. 3, pp. 187-199.
- Lipsitch, M. 2001, "The rise and fall of antimicrobial resistance", *Trends Microbiol.*, vol. 9, pp. 438-444.
- Little, J. W., Kim, B., Roland, K. L., Smith, M. H., Lin, L. L., & Slilaty, S. N. 1994, "Cleavage of LexA repressor", *Methods Enzymol.*, vol. 244, pp. 266-284.
- Little, J. W. & Mount, D. W. 1982, "The SOS regulatory system of *Escherichia coli*", *Cell*, vol. 29, no. 1, pp. 11-22.
- Little, J. W., Mount, D. W., & Yanisch-Perron, C. R. 1981, "Purified *lexA* protein is a repressor of the *recA* and *lexA* genes", *Proc.Natl.Acad.Sci.U.S.A*, vol. 78, no. 7, pp. 4199-4203.
- Liu, J., Takiff, H. E., & Nikaido, H. 1996, "Active efflux of fluoroquinolones in *Mycobacterium smegmatis* mediated by LfrA, a multidrug efflux pump", *The Journal of Bacteriology*, vol. 178, no. 13, pp. 3791-3795.
- Livak, K. J. & Schmittgen, T. D. 2001, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method", *Methods*, vol. 25, no. 4, pp. 402-408.



- Llewelyn, M., Cropley, I., Wilkinson, R. J., & Davidson, R. N. 2000, "Tuberculosis diagnosed during pregnancy: a prospective study from London", *Thorax*, vol. 55, no. 2, pp. 129-132.
- Lonetto, M., Gribskov, M., & Gross, C. A. 1992, "The sigma 70 family: sequence conservation and evolutionary relationships", *The Journal of Bacteriology*, vol. 174, no. 12, pp. 3843-3849.
- Long, R. 2000, "Drug-resistant tuberculosis", *Canadian Medical Association Journal*, vol. 163, no. 4, pp. 425-428.
- Lounis, N., Bentoucha, A., Truffot-Pernot, C., Ji, B., O'Brien, R. J., Vernon, A., Roscigno, G., & Grosset, J. 2001, "Effectiveness of Once-Weekly Rifapentine and Moxifloxacin Regimens against *Mycobacterium tuberculosis* in Mice", *Antimicrobial Agents and Chemotherapy*, vol. 45, no. 12, pp. 3482-3486.
- Lubasch, A., Erbes, R., Mauch, H., & Lode, H. 2001, "Sparfloxacin in the treatment of drug resistant tuberculosis or intolerance of first line therapy", *European Respiratory Journal*, vol. 17, no. 4, pp. 641-646.
- Lubasch, A., Keller, I., Borner, K., Koeppe, P., & Lode, H. 2000, "Comparative pharmacokinetics of ciprofloxacin, gatifloxacin, grepafloxacin, levofloxacin, trovafloxacin, and moxifloxacin after single oral administration in healthy volunteers", *Antimicrobial Agents and Chemotherapy*, vol. 44, no. 10, pp. 2600-2603.
- Luna-Herrera, J., Reddy, M. V., & Gangadharam, P. R. 1995, "In-vitro and intracellular activity of rifabutin on drug-susceptible and multiple drug-resistant (MDR) tubercle bacilli", *Journal of Antimicrobial Chemotherapy*, vol. 36, no. 2, pp. 355-363.
- Luria, S. & Delbrück, M. 1943, "Mutations of bacteria from virus sensitivity to virus resistance.", *Genetics*, vol. 28, pp. 491-511.
- Lurie, M. B. 1964, *Resistance to Tuberculosis: Experimental Studies in Native and Acquired Defensive Mechanisms*. Harvard University Press, Cambridge, Mass.

- Luttinger, A. 1995, "The twisted 'life' of DNA in the cell: bacterial topoisomerases", *Mol. Microbiol.*, vol. 15, no. 4, pp. 601-606.
- Lynch, M., Conery, J., & Burger, R. 1995, "Mutant Accumulation and the Extinction of Small Populations", *American Naturalist*, vol. 146, pp. 489-518.
- Mackay, W. J., Han, S., & Samson, L. D. 1994, "DNA alkylation repair limits spontaneous base substitution mutations in *Escherichia coli*", *The Journal of Bacteriology*, vol. 176, no. 11, pp. 3224-3230.
- Maguire, H., Dale, J. W., McHugh, T. D., Butcher, P. D., Gillespie, S. H., Costetsos, A., Al Ghusein, H., Holland, R., Dickens, A., Marston, L., Wilson, P., Pitman, R., Strachan, D., Drobniewski, F. A., & Banerjee, D. K. 2002, "Molecular epidemiology of tuberculosis in London 1995-7 showing low rate of active transmission", *Thorax*, vol. 57, no. 7, pp. 617-622.
- Maisnier-Patin, S. & Andersson, D. I. 2004, "Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution", *Research in Microbiology*, vol. 155, pp. 360-369.
- Maisnier-Patin, S., Berg, O. G., Liljas, L., & Andersson, D. I. 2002, "Compensatory adaptation to the deleterious effect of antibiotic resistance in *Salmonella typhimurium*", *Molecular Microbiology*, vol. 46, no. 2, pp. 355-366.
- Mamber, S. W., Kolek, B., Brookshire, K. W., Bonner, D. P., & Fung-Tomc, J. 1993, "Activity of quinolones in the Ames Salmonella TA102 mutagenicity test and other bacterial genotoxicity assays", *Antimicrobial Agents and Chemotherapy*, vol. 37, no. 2, pp. 213-217.
- Manca, C., Paul, S., Barry, C. E., III, Freedman, V. H., & Kaplan, G. 1999, "*Mycobacterium tuberculosis* catalase and peroxidase activities and resistance to oxidative killing in human monocytes *in vitro*", *Infection and Immunity*, vol. 67, no. 1, pp. 74-79.

- Mangan, J. A., Monahan, I. M., & Butcher, P. D. 2002, "Gene expression during host-pathogen interactions: approaches to bacterial mRNA extraction and labelling for microarray analysis," in *Vol 33 Functional Microbial Genomics: Methods in Microbiology*, B. Wren & N. Dorrell, eds., Academic Press, London, pp. 137-151.
- Manganelli, R., Dubnau, E., Tyagi, S., Kramer, F. R., & Smith, I. 1999, "Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*", *Molecular Microbiology*, vol. 31, pp. 715-724.
- Manganelli, R., Voskuil, M. I., Schoolnik, G. K., Dubnau, E., Gomez, M., & Smith, I. 2002, "Role of the extracytoplasmic-function Factor H in *Mycobacterium tuberculosis* global gene expression", *Molecular Microbiology*, vol. 45, no. 2, pp. 365-374.
- Mani, C., Selvakumar, N., Narayanan, S., & Narayanan, P. R. 2001, "Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* clinical isolates from India", *Journal of Clinical Microbiology*, vol. 39, no. 8, pp. 2987-2990.
- March, F., Garriga, X., Rodriguez, P., Moreno, C., Garrigo, M., Coll, P., & Prats, G. 1997, "Acquired drug resistance in *Mycobacterium tuberculosis* isolates recovered from compliant patients with human immunodeficiency virus-associated tuberculosis", *Clin.Infect.Dis.*, vol. 25, no. 5, pp. 1044-1047.
- Mariam, D. H., Mengistu, Y., Hoffner, S. E., & Andersson, D. I. 2004, "Effect of *rpoB* mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis*", *Antimicrobial Agents and Chemotherapy*, vol. 48, no. 4, pp. 1289-1294.
- Marrero, A., Caminero, J. A., Rodriguez, R., & Billo, N. E. 2000, "Towards elimination of tuberculosis in a low income country: the experience of Cuba, 1962-97", *Thorax*, vol. 55, no. 1, pp. 39-45.
- Martinez, J. L. & Baquero, F. 2000, "Mutation frequencies and antibiotic resistance", *Antimicrobial Agents and Chemotherapy*, vol. 44, no. 7, pp. 1771-1777.

- Matsiota-Bernard, P., Vrioni, G., & Marinis, E. 1998, "Characterization of *rpoB* mutations in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates from Greece", *Journal of Clinical Microbiology*, vol. 36, no. 1, pp. 20-23.
- Marttila, H. J., Soini, H., Vyshnevskaya, E., Vyshnevskiy, B. I., Otten, T. F., Vasilyef, A. V., & Viljanen, M. K. 1999, "Line probe assay in the rapid detection of rifampin-resistant *Mycobacterium tuberculosis* directly from clinical specimens", *Scand.J.Infect.Dis.*, vol. 31, no. 3, pp. 269-273.
- Master, S. S., Springer, B., Sander, P., Boettger, E. C., Deretic, V., & Timmins, G. S. 2002, "Oxidative stress response genes in *Mycobacterium tuberculosis*: role of *ahpC* in resistance to peroxynitrite and stage-specific survival in macrophages", *Microbiology*, vol. 148, no. 10, pp. 3139-3144.
- Matsunaga, I., Oka, S., Inoue, T., & Yano, I. 1990, "Mycolyl glycolipids stimulate macrophages to release a chemotactic factor", *FEMS Microbiology Letters*, vol. 67, no. 1-2, pp. 49-53.
- McAdam, R. A., Hermans, P. W., Van Soolingen, D., Zainuddin, Z. F., Catty, D., van Embden, J. D., & Dale, J. W. 1990, "Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS3 family", *Mol.Microbiol.*, vol. 4, no. 9, pp. 1607-1613.
- McCarter, Y. S., Ratkiewicz, I. N., & Robinson, A. 1998, "Cord Formation in BACTEC Medium Is a Reliable, Rapid Method for Presumptive Identification of *Mycobacterium tuberculosis* Complex", *Journal of Clinical Microbiology*, vol. 36, no. 9, pp. 2769-2771.
- McClatchy, J. K., Tsang, A. Y., & Cernich, M. S. 1981, "Use of pyrazinamidase activity on *Mycobacterium tuberculosis* as a rapid method for determination of pyrazinamide susceptibility", *Antimicrobial Agents and Chemotherapy*, vol. 20, no. 4, pp. 556-557.
- McClure, W. R. & Cech, C. L. 1978, "On the mechanism of rifampicin inhibition of RNA synthesis", *Journal of Biological Chemistry*, vol. 253, no. 24, pp. 8949-8956.

- McDermott, W. & Tompsett, R. 1954, "Activation of pyrazinamide and nicotinamide in acidic environments *in vitro*", *Am.Rev.Tuberc.*, vol. 70, no. 4, pp. 748-754.
- McFadden, J. 1996, "Recombination in mycobacteria", *Molecular Microbiology*, vol. 21, no. 2, pp. 205-211.
- McGarvey, J. A., Wagner, D., & Bermudez, L. E. 2004, "Differential gene expression in mononuclear phagocytes infected with pathogenic and non-pathogenic mycobacteria", *Clin.Exp.Immunol.*, vol. 136, no. 3, pp. 490-500.
- McKenzie, G. J., Harris, R. S., Lee, P. L., & Rosenberg, S. M. 2000, "The SOS response regulates adaptive mutation", *Proceedings of the National Academy of Sciences*, vol. 97, no. 12, pp. 6646-6651.
- McKinney, J. D., Hoener Zu Bentrup, K., Munoz-Elias, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs Jr, W. R., & Russell, D. G. 2002, "Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase", *Nature*, vol. 406, pp. 735-738.
- McNeil, M. R. & Brennan, P. J. 1991, "Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis and drug resistance; some thoughts and possibilities arising from recent structural information", *Res.Microbiol.*, vol. 142, no. 4, pp. 451-463.
- McPartland, A., Green, L., & Echols, H. 1980, "Control of *recA* gene RNA in *E. coli*: regulatory and signal genes", *Cell*, vol. 20, no. 3, pp. 731-737.
- Meachen, N. G. 1936, *A short history of tuberculosis*. Staples Press Ltd., London.
- Meier, A., Sander, P., Schaper, K. J., Scholz, M., & Bottger, E. C. 1996, "Correlation of molecular resistance mechanisms and phenotypic resistance levels in streptomycin-resistant *Mycobacterium tuberculosis*", *Antimicrobial Agents and Chemotherapy*, vol. 40, no. 11, pp. 2452-2454.

## References

- Mestdagh, M., Fonteyne, P. A., Realini, L., Rossau, R., Jannes, G., Mijs, W., De Smet, K. A., Portaels, F., & Van den, E. E. 1999, "Relationship between pyrazinamide resistance, loss of pyrazinamidase activity, and mutations in the *pncA* locus in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis*", *Antimicrobial Agents and Chemotherapy*, vol. 43, no. 9, pp. 2317-2319.
- Michaels, M. L., Cruz, C., Grollman, A. P., & Miller, J. H. 1992, "Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA", *Proceedings of the National Academy of Sciences of the USA*, vol. 89, no. 15, pp. 7022-7025.
- Middlebrook, G. 1952, "Sterilization of tubercle bacilli by isonicotinic acid hydrazide and the incidence of variants resistant to the drug *in vitro*", *Am.Rev.Tuberc.*, vol. 65, no. 6, pp. 765-767.
- Middlebrook, G. & Cohn, M. L. 1953, "Some observations on the pathogenicity of isoniazid resistant variants of tubercle bacilli", *Science*, vol. 118, pp. 297-299.
- Middlebrook, G. 1954, "Isoniazid-resistance and catalase activity of tubercle bacilli; a preliminary report", *American Review of Tuberculosis*, vol. 69, no. 3, pp. 471-472.
- Middlebrook, G., Dubos, R. J., & Pierce, C. 1947, "Virulence and Morphological Characteristics of mammalian tubercle bacilli.", *The Journal of Experimental Medicine*, vol. 86, no. 2, pp. 175-184.
- Miesel, L., Weisbrod, T. R., Marcinkeviciene, J. A., Bittman, R., & Jacobs, W. R., Jr. 1998, "NADH Dehydrogenase Defects Confer Isoniazid Resistance and Conditional Lethality in *Mycobacterium smegmatis*", *The Journal of Bacteriology*, vol. 180, no. 9, pp. 2459-2467.
- Migliori, G. B., Espinal, M., Danilova, I. D., Punga, V. V., Grzemska, M., & Raviglione, M. C. 2002, "Frequency of recurrence among MDR-TB cases 'successfully' treated with

- standardised short-course chemotherapy", *Int.J.Tuberc.Lung Dis.*, vol. 6, no. 10, pp. 858-864.
- Miles, A. & Misra, S. 1938, "The estimation of the bacteriocidal power of blood", *J Hyg (Camb)*, vol. 38, pp. 732-749.
- Miller, J. H. & Low, K. B. 1984, "Specificity of mutagenesis resulting from the induction of the SOS system in the absence of mutagenic treatment", *Cell*, vol. 37, no. 2, pp. 675-682.
- Mitchison, D. A. 1979, "Basic mechanisms of chemotherapy", *Chest*, vol. 76, no. 6 Suppl, pp. 771-781.
- Mitchison, D. A. 1985, "The action of antituberculosis drugs in short-course chemotherapy", *Tubercle.*, vol. 66, no. 3, pp. 219-225.
- Mitchison, D. A. 1993, "Assessment of new sterilizing drugs for treating pulmonary tuberculosis by culture at 2 months", *Am.Rev.Respir.Dis.*, vol. 147, no. 4, pp. 1062-1063.
- Mitchison, D. A. 1997, "Mechanisms of tuberculosis chemotherapy", *J.Pharm.Pharmacol.*, vol. 49, no. suppl.1, pp. 31-36.
- Mitchison, D. A., Wallace, J. G., Bhatia, A. L., Selkon, J. B., & Lancaster, M. C. 1960, "A comparison of the virulence in guinea pigs of South India and British tubercle bacilli", *Tubercle*, vol. 41, pp. 1-22.
- Mizrahi, V. & Andersen, S. J. 1998, "DNA repair in *Mycobacterium tuberculosis*. What have we learnt from the genome sequence?", *Molecular Microbiology*, vol. 29, no. 6, pp. 1331-1339.
- Moghazeh, S. L., Pan, X., Arain, T., Stover, C. K., Musser, J. M., & Kreiswirth, B. N. 1996, "Comparative antimycobacterial activities of rifampin, rifapentine, and KRM-1648 against a collection of rifampin-resistant *Mycobacterium tuberculosis* isolates with

- known *rpoB* mutations", *Antimicrobial Agents and Chemotherapy*, vol. 40, no. 11, pp. 2655-2657.
- Mokrousov, I., Narvskaya, O., Limeschenko, E., Otten, T., & Vyshnevskiy, B. 2002, "Detection of ethambutol-resistant *Mycobacterium tuberculosis* strains by multiplex allele-specific PCR assay targeting *embB306* mutations", *Journal of Clinical Microbiology*, vol. 40, no. 5, pp. 1617-1620.
- Mokrousov, I., Otten, T., Vyshnevskiy, B., & Narvskaya, O. 2003, "Allele-specific *rpoB* PCR assays for detection of rifampin-resistant *Mycobacterium tuberculosis* in sputum smears", *Antimicrobial Agents and Chemotherapy*, vol. 47, no. 7, pp. 2231-2235.
- Mondon, P., Petter, R., Amalfitano, G., Luzzati, R., Concia, E., Polacheck, I., & Kwon-Chung, K. J. 1999, "Heteroresistance to fluconazole and voriconazole in *Cryptococcus neoformans*", *Antimicrobial Agents and Chemotherapy*, vol. 43, no. 8, pp. 1856-1861.
- Moore, F. B., Rozen, D. E., & Lenski, R. E. 2000, "Pervasive compensatory adaptation in *Escherichia coli*", *Proc.R.Soc.Lond B Biol.Sci.*, vol. 267, no. 1442, pp. 515-522.
- Morlock, G. P., Plikaytis, B. B., & Crawford, J. T. 2000, "Characterization of spontaneous, *In vitro*-selected, rifampin-resistant mutants of *Mycobacterium tuberculosis* strain H37Rv", *Antimicrobial Agents and Chemotherapy*, vol. 44, no. 12, pp. 3298-3301.
- Morris, S., Bai, G. H., Suffys, P., Portillo-Gomez, L., Fairchok, M., & Rouse, D. 1995, "Molecular mechanisms of multiple drug resistance in clinical isolates of *Mycobacterium tuberculosis*", *Journal of Infectious Diseases*, vol. 171, no. 4, pp. 954-960.
- Morris, R. P., Nguyen, L., Gatfield, J., Visconti, K., Nguyen, K., Schnappinger, D., Ehrt, S., Liu, Y., Heifets, L., Pieters, J., Schoolnik, G., & Thompson, C. J. 2005, "Ancestral antibiotic resistance in *Mycobacterium tuberculosis*", *Proceedings of the National Academy of Sciences*, vol. 102, no. 34, pp. 12200-12205.
- Mostowy, S., Cousins, D., Brinkman, J., Aranaz, A., & Behr, M. A. 2002, "Genomic deletions suggest a phylogeny for the *Mycobacterium tuberculosis* complex", *J.Infect.Dis.*, vol. 186, no. 1, pp. 74-80.



- Movahedzadeh, F., Colston, M. J., & Davis, E. O. 1997a, "Characterization of *Mycobacterium tuberculosis* LexA: recognition of a Cheo (Bacillus-type SOS) box", *Microbiology*, vol. 143, no. 3, pp. 929-936.
- Movahedzadeh, F., Colston, M. J., & Davis, E. O. 1997b, "Determination of DNA sequences required for regulated *Mycobacterium tuberculosis* RecA expression in response to DNA-damaging agents suggests that two modes of regulation exist", *The Journal of Bacteriology*, vol. 179, no. 11, pp. 3509-3518.
- Murray, M. B. 2002, "Molecular epidemiology and the dynamics of tuberculosis transmission among foreign-born people", *Canadian Medical Association Journal*, vol. 167, no. 4, pp. 355-356.
- Musser, J. M. 1995, "Antimicrobial agent resistance in mycobacteria: molecular genetic insights", *Clinical Microbiology Reviews*, vol. 8, no. 4, pp. 496-514.
- Musser, J. M., Kapur, V., Williams, D. L., Kreiswirth, B. N., Van Soolingen, D., & van Embden, J. D. 1996, "Characterization of the catalase-peroxidase gene (*katG*) and *inhA* locus in isoniazid-resistant and -susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: restricted array of mutations associated with drug resistance", *Journal of Infectious Diseases*, vol. 173, no. 1, pp. 196-202.
- Musser, J. M., Amin, A., & Ramaswamy, S. 2000, "Negligible Genetic Diversity of *Mycobacterium tuberculosis* Host Immune System Protein Targets: Evidence of Limited Selective Pressure", *Genetics*, vol. 155, no. 1, pp. 7-16.
- Nachamkin, I., Kang, C., & Weinstein, M. P. 1997, "Detection of resistance to isoniazid, rifampin, and streptomycin in clinical isolates of *Mycobacterium tuberculosis* by molecular methods", *Clinical Infectious Diseases*, vol. 24, no. 5, pp. 894-900.
- Nakamura, Y., Noma, M., Kidokoro, M., Kobayashi, N., Takei, M., Kurashima, S., Mukaiyama, T., & Kato, S. 1994, "Isolation of human CD34<sup>+</sup> peripheral blood stem cells using an immunomagnetic positive selection system: prior monocyte depletion using a nylon-wool column", *J Hematother.*, vol. 3, no. 2, pp. 128-133.

## References

- Nadon, R., Shi, P., Skandalis, A., Woody, E., Hubschle, H., Susko, E., Rghei, N., & Ramm, P. 2001, "Statistical inference methods for gene expression arrays", *Proceedings of SPIE, BIOS, Microarrays, Optical Technologies and Informatics* pp. 46-55.
- Narberhaus, F. 1999, "Negative regulation of bacterial heat shock genes", *Molecular Microbiology*, vol. 31, pp. 1-8.
- Nau, G. J., Richmond, J. F., Schlesinger, A., Jennings, E. G., Lander, E. S., & Young, R. A. 2002, "Human macrophage activation programs induced by bacterial pathogens", *Proc.Natl.Acad.Sci.U.S.A*, vol. 99, no. 3, pp. 1503-1508.
- Nau, G. J., Schlesinger, A., Richmond, J. F., & Young, R. A. 2003, "Cumulative Toll-like receptor activation in human macrophages treated with whole bacteria", *J.Immunol.*, vol. 170, no. 10, pp. 5203-5209.
- Nghiem, Y., Cabrera, M., Cupples, C. G., & Miller, J. H. 1988, "The *mutY* gene: a mutator locus in *Escherichia coli* that generates G.C----T.A transversions", *Proceedings of the National Academy of Sciences of the USA*, vol. 85, no. 8, pp. 2709-2713.
- Nguyen, L. N., Gilbert, G. L., & Marks, G. B. 2004, "Molecular epidemiology of tuberculosis and recent developments in understanding the epidemiology of tuberculosis", *Respirology*, vol. 9, no. 3, pp. 313-319.
- Niimura, Y., Poole, L. B., & Massey, V. 1995, "*Amphibacillus xylanus* NADH oxidase and *Salmonella typhimurium* alkyl-hydroperoxide reductase flavoprotein components show extremely high scavenging activity for both alkyl hydroperoxide and hydrogen peroxide in the presence of *S. typhimurium* alkyl-hydroperoxide reductase 22-kDa protein component", *Journal of Biological Chemistry*, vol. 270, no. 43, pp. 25645-25650.
- Nikaido, H. 1994, "Prevention of drug access to bacterial targets: permeability barriers and active efflux", *Science*, vol. 264, no. 5157, pp. 382-388.
- Nikaido, H. 1996, "Multidrug efflux pumps of gram-negative bacteria", *The Journal of Bacteriology*, vol. 178, no. 20, pp. 5853-5859.

## References

- Nitta, A. T., Knowles, L. S., Kim, J., Lehnkering, E. L., Borenstein, L. A., Davidson, P. T., Harvey, S. M., & De Koning, M. L. 2002, "Limited transmission of multidrug-resistant tuberculosis despite a high proportion of infectious cases in Los Angeles County, California", *Am.J.Respir.Crit Care Med*, vol. 165, no. 6, pp. 812-817.
- Novotna, J., Vohradsky, J., Berndt, P., Gramajo, H., Langen, H., Li, X. M., Minas, W., Orsaria, L., Roeder, D., & Thompson, C. J. 2003, "Proteomic studies of diauxic lag in the differentiating prokaryote *Streptomyces coelicolor* reveal a regulatory network of stress-induced proteins and central metabolic enzymes", *Molecular Microbiology*, vol. 48, no. 5, pp. 1289-1303.
- Nurse, P., Zavitz, K. H., & Marians, K. J. 1991, "Inactivation of the *Escherichia coli* *priA* DNA replication protein induces the SOS response", *The Journal of Bacteriology*, vol. 173, no. 21, pp. 6686-6693.
- Ogawa, H. & Ogawa, T. 1990, "Regulation in repressor inactivation by RecA protein", *Adv.Biophys.*, vol. 26, pp. 33-49.
- Ohno, H., Koga, H., Kohno, S., Tashiro, T., & Hara, K. 1996, "Relationship between rifampin MICs for and *rpoB* mutations of *Mycobacterium tuberculosis* strains isolated in Japan", *Antimicrobial Agents and Chemotherapy*, vol. 40, no. 4, pp. 1053-1056.
- Onodera, Y., Tanaka, M., & Sato, K. 2001, "Inhibitory activity of quinolones against DNA gyrase of *Mycobacterium tuberculosis*", *Journal of Antimicrobial Chemotherapy*, vol. 47, no. 4, pp. 447-450.
- Opie, E. L. & Aronson, J. D. 1927, "Tubercle bacilli in latent tuberculous lesions and in lung tissue without tuberculous lesions.", *Arch.Path.Lab.Med.*, vol. 4, p. 1.
- Ordway, D. J., Sonnenberg, M. G., Donahue, S. A., Belisle, J. T., & Orme, I. M. 1995, "Drug-resistant strains of *Mycobacterium tuberculosis* exhibit a range of virulence for mice", *Infect.Immun.*, vol. 63, no. 2, pp. 741-743.
- Orellana, C. 2002, "Russia learns to cope with tuberculosis", *The Lancet Infectious Diseases*, vol. 2, p. 324.

- Ormerod, L. P. 2003, "Non-respiratory tuberculosis," in *Clinical Tuberculosis*, 3 edn, P. D. O. Davies, ed., Arnold Publishers, London, pp. 125-153.
- Pablos-Mendez, A., Raviglione, M. C., Laszlo, A., Binkin, N., Rieder, H. L., Bustreo, F., Cohn, D. L., Lambregts-van Weezenbeek, C. S., Kim, S. J., Chaulet, P., & Nunn, P. 1998, "Global surveillance for antituberculosis-drug resistance, 1994-1997. World Health Organization-International Union against Tuberculosis and Lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance", *N.Engl.J.Med.*, vol. 338, no. 23, pp. 1641-1649.
- Palmer, C. E., Jablon, S., & Edwards, P. Q. 1957, "Tuberculosis morbidity of young men in relation to tuberculin sensitivity and body build", *Am.Rev.Tuberc.*, vol. 76, no. 4, pp. 517-539.
- Papavinasundaram, K. G., Anderson, C., Brooks, P. C., Thomas, N. A., Movahedzadeh, F., Jenner, P. J., Colston, M. J., & Davis, E. O. 2001, "Slow induction of RecA by DNA damage in *Mycobacterium tuberculosis*", *Microbiology*, vol. 147, pp. 3271-3279.
- Papavinasundaram, K. G., Movahedzadeh, F., Keer, J. T., Stoker, N. G., Colston, M. J., & Davis, E. O. 1997, "Mycobacterial *recA* is cotranscribed with a potential regulatory gene called *recX*", *Mol.Microbiol.*, vol. 24, no. 1, pp. 141-153.
- Parrish, N. M., Dick, J. D., & Bishai, W. R. 1998, "Mechanisms of latency in *Mycobacterium tuberculosis*", *Trends in Microbiology*, vol. 6, no. 3, pp. 107-112.
- Paulsen, I. T., Brown, M. H., & Skurray, R. A. 1996, "Proton-dependent multidrug efflux systems", *Microbiology and Molecular Biology Reviews*, vol. 60, no. 4, pp. 575-608.
- Perelman, M. I. 2000, "Tuberculosis in Russia", *Int.J.Tuberc.Lung Dis.*, vol. 4, no. 12, pp. 1097-1103.
- Perlman, D. C., El Sadr, W. M., Heifets, L. B., Nelson, E. T., Matts, J. P., Chirgwin, K., Salomon, N., Telzak, E. E., Klein, O., Kreiswirth, B. N., Musser, J. M., & Hafner, R. 1997, "Susceptibility to levofloxacin of *Mycobacterium tuberculosis* isolates from patients with HIV-related tuberculosis and characterization of a strain with levofloxacin

monoresistance. Community Programs for Clinical Research on AIDS 019 and the AIDS Clinical Trials Group 222 Protocol Team", *AIDS*, vol. 11, no. 12, pp. 1473-1478.

Peters, W. 1987, *Chemotherapy and drug resistance in malaria*, 2nd edn, Academic Press, London.

Peterson, K. R. & Mount, D. W. 1993, "Analysis of the genetic requirements for viability of *Escherichia coli* K-12 DNA adenine methylase (dam) mutants", *The Journal of Bacteriology*, vol. 175, no. 22, pp. 7505-7508.

Peterson, K. R., Ossanna, N., Thliveris, A. T., Ennis, D. G., & Mount, D. W. 1988, "Derepression of specific genes promotes DNA repair and mutagenesis in *Escherichia coli*", *The Journal of Bacteriology*, vol. 170, no. 1, pp. 1-4.

Peterson, K. R., Wertman, K. F., Mount, D. W., & Marinus, M. G. 1985, "Viability of *Escherichia coli* K-12 DNA adenine methylase (dam) mutants requires increased expression of specific genes in the SOS regulon", *Mol.Gen.Genet.*, vol. 201, no. 1, pp. 14-19.

Petrini, B. & Hoffner, S. 1999, "Drug-resistant and multidrug-resistant tubercle bacilli", *International Journal of Antimicrobial Agents*, vol. 13, no. 93, p. 97.

Philipp, W. J., Poulet, S., Eiglmeier, K., Pascopella, L., Balasubramanian, V., Heym, B., Bergh, S., Bloom, B. R., Jacobs, W. R., Jr., & Cole, S. T. 1996, "An integrated map of the genome of the tubercle bacillus, *Mycobacterium tuberculosis* H37Rv, and comparison with *Mycobacterium leprae*", *Proceedings of the National Academy of Sciences*, vol. 93, no. 7, pp. 3132-3137.

Phillips, I., Culebras, E., Moreno, F., & Baquero, F. 1987, "Induction of the SOS response by new 4-quinolones", *J Antimicrob.Chemother.*, vol. 20, no. 5, pp. 631-638.

Piatek, A. S., Telenti, A., Murray, M. R., El Hajj, H., Jacobs, W. R., Jr., Kramer, F. R., & Alland, D. 2000, "Genotypic Analysis of *Mycobacterium tuberculosis* in Two Distinct Populations Using Molecular Beacons: Implications for Rapid Susceptibility Testing", *Antimicrobial Agents and Chemotherapy*, vol. 44, no. 1, pp. 103-110.

- Pickup, R. W., Rhodes, G., Arnott, S., Sidi-Boumedine, K., Bull, T. J., Weightman, A., Hurley, M., & Hermon-Taylor, J. 2005, "*Mycobacterium avium* subsp. *paratuberculosis* in the Catchment Area and Water of the River Taff in South Wales, United Kingdom, and Its Potential Relationship to Clustering of Crohn's Disease Cases in the City of Cardiff", *Applied and Environmental Microbiology*, vol. 71, no. 4, pp. 2130-2139.
- Piddock, L. J., Hall, M. C., & Walters, R. N. 1991, "Phenotypic characterization of quinolone-resistant mutants of Enterobacteriaceae selected from wild type, *gyrA* type and multiply-resistant (*marA*) type strains", *Journal of Antimicrobial Chemotherapy*, vol. 28, no. 2, pp. 185-198.
- Piddock, L. J. V., Williams, K. J., & Ricci, V. 2000, "Accumulation of rifampicin by *Mycobacterium aurum*, *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*", *Journal of Antimicrobial Chemotherapy*, vol. 45, no. 2, pp. 159-165.
- Piddock, L. J. V. & Wise, R. 1987, "Induction of the SOS response in *Escherichia coli* by 4-quinolone antibacterial agents", *FEMS Microbiology Letters*, vol. 41, pp. 289-294.
- Pio, A. & Chaulet, P. 1998, *Tuberculosis Handbook*, WHO, Geneva.
- Pitulle, C., Dorsch, M., Kazda, J., Wolters, J., & Stackebrandt, E. 1992, "Phylogeny of rapidly growing members of the genus *Mycobacterium*", *Int.J.Syst.Bacteriol.*, vol. 42, no. 3, pp. 337-343.
- Poulet, S. & Cole, S. T. 1995a, "Characterization of the highly abundant polymorphic GC-rich-repetitive sequence (PGRS) present in *Mycobacterium tuberculosis*", *Arch.Microbiol.*, vol. 163, no. 2, pp. 87-95.
- Poulet, S. & Cole, S. T. 1995b, "Repeated DNA sequences in mycobacteria", *Arch Microbiol.*, vol. 163, no. 2, pp. 79-86.
- Poulsen, A. 1954, "Some clinical features of tuberculosis", *Acta.Tuberc.Scand.*, vol. 24, no. 16, pp. 311-346.

Power, E. G. & Phillips, I. 1992, "Induction of the SOS gene (*umuC*) by 4-quinolone antibacterial drugs", *J.Med.Microbiol.*, vol. 36, no. 2, pp. 78-82.

Power, E. G. & Phillips, I. 1993, "Correlation between *umuC* induction and Salmonella mutagenicity assay for quinolone antimicrobial agents", *FEMS Microbiol.Lett.*, vol. 112, no. 3, pp. 251-254.

Pozzi, G., Meloni, M., Iona, E., Orru, G., Thoresen, O. F., Ricci, M. L., Oggioni, M. R., Fattorini, L., & Orefici, G. 1999, "*rpoB* mutations in multidrug-resistant strains of *Mycobacterium tuberculosis* isolated in Italy", *Journal of Clinical Microbiology*, vol. 37, no. 4, pp. 1197-1199.

Pretorius, G. S., van Helden, P. D., Sirgel, F., Eisenach, K. D., & Victor, T. C. 1995, "Mutations in *katG* gene sequences in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* are rare", *Antimicrobial Agents and Chemotherapy*, vol. 39, no. 10, pp. 2276-2281.

Proudfoot, A. T., Akhtar, A. J., Douglas, A. C., & Horne, N. W. 1969, "Miliary tuberculosis in adults", *Br.Med J*, vol. 2, no. 652, pp. 273-276.

Purmal, A. A., Kow, Y. W., & Wallace, S. S. 1994a, "5-Hydroxypyrimidine deoxynucleoside triphosphates are more efficiently incorporated into DNA by exonuclease-free Klenow fragment than 8-oxopurine deoxynucleoside triphosphates", *Nucleic Acids Research*, vol. 22, no. 19, pp. 3930-3935.

Purmal, A. A., Kow, Y. W., & Wallace, S. S. 1994b, "Major oxidative products of cytosine, 5-hydroxycytosine and 5-hydroxyuracil, exhibit sequence context-dependent mispairing *in vitro*", *Nucleic Acids Research*, vol. 22, no. 1, pp. 72-78.

Pym, A. S., Domenech, P., Honoré, N., Song, J., Deretic, V., & Cole, S. 2001, "Regulation of catalase-peroxidase (KatG) expression, isoniazid sensitivity and virulence by *furA* of *Mycobacterium tuberculosis*", *Molecular Microbiology*, vol. 40, no. 4, pp. 879-889.

- Pym, A. S., Saint-Joanis, B., & Cole, S. T. 2002, "Effect of *katG* mutations on the virulence of *Mycobacterium tuberculosis* and the implication for transmission in humans", *Infection and Immunity*, vol. 70, no. 9, pp. 4955-4960.
- Rad, M. E., Bifani, P., Martin, C., Kremer, K., Samper, S., Rauzier, J., Kreiswirth, B., Blazquez, J., Jouan, M., van Soolingen, D., & Gicquel, B. 2003, "Mutations in putative mutator genes of *Mycobacterium tuberculosis* strains of the W-Beijing family", *Emerg.Infect.Dis.*, vol. 9, no. 7, pp. 838-845.
- Radman, M. 1974, "Phenomenology of an inducible mutagenic DNA repair pathway in *Escherichia coli*: SOS repair hypothesis," in *Molecular and Environmental Aspects of Mutagenesis*, S. Sherman et al., eds., Charles C. Thomas, Springfield, pp. 128-142.
- Radman, M. 1998, "DNA replication: one strand may be more equal", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 95, no. 17, pp. 9718-9719.
- Ramaswamy, S. & Musser, J. M. 1998, "Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update", *Tubercle and Lung Disease*, vol. 79, no. 1, pp. 3-29.
- Ramaswamy, S. V., Dou, S. J., Rendon, A., Yang, Z., Cave, M. D., & Graviss, E. A. 2004, "Genotypic analysis of multidrug-resistant *Mycobacterium tuberculosis* isolates from Monterrey, Mexico", *Journal of Medical Microbiology*, vol. 53, no. Pt 2, pp. 107-113.
- Rand, L., Hinds, J., Springer, B., Sander, P., Buxton, R. S., & Davis, E. O. 2003, "The majority of inducible DNA repair genes in *Mycobacterium tuberculosis* are induced independently of RecA", *Molecular Microbiology*, vol. 50, no. 3, pp. 1031-1042.
- Rastogi, N., Labrousse, V., & Goh, K. S. 1996, "In vitro activities of fourteen antimicrobial agents against drug susceptible and resistant clinical isolates of *Mycobacterium tuberculosis* and comparative intracellular activities against the virulent H37Rv strain in human macrophages", *Curr.Microbiol.*, vol. 33, no. 3, pp. 167-175.



- Rastogi, N., Legrand, E., & Sola, C. 2001, "The mycobacteria: an introduction to nomenclature and pathogenesis", *Rev.Sci.Tech.*, vol. 20, no. 1, pp. 21-54.
- Rastogi, N., Ross, B. C., Dwyer, B., Goh, K. S., Clavel-Seres, S., Jeantils, V., & Cruaud, P. 1992, "Emergence during unsuccessful chemotherapy of multiple drug resistance in a strain of *Mycobacterium tuberculosis*", *Eur J Clin.Microbiol.Infect.Dis.*, vol. 11, no. 10, pp. 901-907.
- Rattan, A., Kalia, A., & Ahmad, N. 1998, "Multidrug-Resistant *Mycobacterium tuberculosis*: Molecular Prespectives", *Emerging Infectious Diseases*, vol. 4, no. 2, pp. 195-209.
- Raviglione, M. C., Dye, C., Schmidt, S., Kochi, A., & and Monitoring Project, f. t. W. G. S. 1997, "Assessment of worldwide tuberculosis control", *The Lancet*, vol. 350, no. 9078, pp. 624-629.
- Raynaud, C., Laneelle, M. A., Senaratne, R. H., Draper, P., Laneelle, G., & Daffe, M. 1999, "Mechanisms of pyrazinamide resistance in mycobacteria: importance of lack of uptake in addition to lack of pyrazinamidase activity", *Microbiology*, vol. 145, no. 6, pp. 1359-1367.
- Reece, R. J. & Maxwell, A. 1991, "DNA gyrase: structure and function", *Critical Reviews in Biochemistry and Molecular Biology*, vol. 26, no. 3, pp. 335-375.
- Reichler, M. R., Reves, R., Bur, S., Thompson, V., Mangura, B. T., Ford, J., Valway, S. E., Onorato, I. M., & for the Contact Investigation Study Group 2002, "Evaluation of Investigations Conducted to Detect and Prevent Transmission of Tuberculosis", *JAMA: The Journal of the American Medical Association*, vol. 287, no. 8, pp. 991-995.
- Renau, T. E., Sanchez, J. P., Shapiro, M. A., Gracheck, S. J., & Domagala, J. M. 1995, "Effect of lipophilicity at N-1 on activity of fluoroquinolones against mycobacteria", *Journal of Medicinal Chemistry*, vol. 38, pp. 2974-2977.
- Reynolds, M. G. 2000, "Compensatory Evolution in Rifampin-Resistant *Escherichia coli*", *Genetics*, vol. 156, no. 4, pp. 1471-1481.

## References

- Rich, E. A., Torres, M., Sada, E., Finegan, C. K., Hamilton, B. D., & Toossi, Z. 1997, "*Mycobacterium tuberculosis* (MTB)-stimulated production of nitric oxide by human alveolar macrophages and relationship of nitric oxide production to growth inhibition of MTB", *Tuber.Lung Dis.*, vol. 78, no. 5-6, pp. 247-255.
- Rieder, H. L. 1999, *Epidemiologic basis of tuberculosis control. International Union Against Tuberculosis and Lung Disease*. Paris.
- Riley, R. L., Mills, C. C., Nyka, W., Weinstock, N., Storey, P. B., Sultan, L. U., Riley, M. C., & Wells, W. F. 1959, "Aerial Dissemination of Pulmonary Tuberculosis. A two-year study of contagion in a tuberculosis ward.", *Am.J.Hyg.*, vol. 70, pp. 185-196.
- Rinder, H., Feldmann, K., Tortoli, E., Grosset, J., Casal, M., Richter, E., Rifai, M., Jarlier, V., Vaquero, M., Rusch-Gerdes, S., Cambau, E., Gutierrez, J., & Loscher, T. 1999, "Culture independent prediction of isoniazid resistance in *Mycobacterium tuberculosis* by *katG* gene analysis directly from sputum samples", *Mol.Diagn.*, vol. 4, pp. 145-152.
- Rinder, H., Mieskes, K. T., & Loscher, T. 2001, "Heteroresistance in *Mycobacterium tuberculosis*", *Int.J.Tuberc.Lung Dis.*, vol. 5, no. 4, pp. 339-345.
- Riska, P. F., Jacobs, W. R., Jr., & Alland, D. 2000, "Molecular determinants of drug resistance in tuberculosis", *Int.J.Tuberc.Lung Dis.*, vol. 4, no. 2 Suppl 1, pp. S4-10.
- Ristow, M., Mohlig, M., Rifai, M., Schatz, H., Feldmann, K., & Pfeiffer, A. 1995, "New isoniazid/ethionamide resistance gene mutation and screening for multidrug-resistant *Mycobacterium tuberculosis* strains", *Lancet*, vol. 346, no. 8973, pp. 502-503.
- Robertson, H. E. 1933, "Persistence of tuberculous infection.", *Am.J.Pathol.*, vol. 9, p. 711.
- Roche, P., Merianos, A., Antic, R., Carnie, J., Christensen, A., Waring, J., Konstantinos, A., Krause, V., Hurwitz, M., Misrachi, A. V., & Bastian, I. 2001, "Tuberculosis notifications in Australia, 1999", *Commun.Dis Intell.*, vol. 25, no. 4, pp. 254-260.

- Rodriguez, G. M., Voskuil, M. I., Gold, B., Schoolnik, G. K., & Smith, I. 2002, "IdeR, an Essential Gene in *Mycobacterium tuberculosis*: Role of IdeR in Iron-Dependent Gene Expression, Iron Metabolism, and Oxidative Stress Response", *Infection and Immunity*, vol. 70, no. 7, pp. 3371-3381.
- Rohrer, S., Maki, H., & Berger-Bachi, B. 2003, "What makes resistance to methicillin heterogeneous?", *J. Med. Microbiol.*, vol. 52, pp. 605-607.
- Rosche, W. A. & Foster, P. L. 2000, "Determining mutation rates in bacterial populations", *Methods*, vol. 20, no. 1, pp. 4-17.
- Rossau, R., Traore, H., De Beenhouwer, H., Mijs, W., Jannes, G., De Rijk, P., & Portaels, F. 1997, "Evaluation of the INNO-LiPA Rif. TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin", *Antimicrobial Agents and Chemotherapy*, vol. 41, no. 10, pp. 2093-2098.
- Rouse, D. A., DeVito, J. A., Li, Z., Byer, H., & Morris, S. L. 1996, "Site-directed mutagenesis of the *katG* gene of *Mycobacterium tuberculosis*: effects on catalase-peroxidase activities and isoniazid resistance", *Mol. Microbiol.*, vol. 22, no. 3, pp. 583-592.
- Rouse, D. A. & Morris, S. L. 1995, "Molecular mechanisms of isoniazid resistance in *Mycobacterium tuberculosis* and *Mycobacterium bovis*", *Infection and Immunity*, vol. 63, no. 4, pp. 1427-1433.
- Ruddy, M. C., Davies, A. P., Yates, M. D., Yates, S., Balasegaram, S., Drabu, Y., Patel, B., Lozewicz, S., Sen, S., Bahl, M., James, E., Lipman, M., Duckworth, G., Watson, J. M., Piper, M., Drobniewski, F. A., & Maguire, H. 2004, "Outbreak of isoniazid resistant tuberculosis in north London", *Thorax*, vol. 59, no. 4, pp. 279-285.
- Russell, D. G., Dant, J., & Sturgill-Koszycki, S. 1996, "*Mycobacterium avium*- and *Mycobacterium tuberculosis*-containing vacuoles are dynamic, fusion-competent vesicles that are accessible to glycosphingolipids from the host cell plasmalemma", *The Journal of Immunology*, vol. 156, no. 12, pp. 4764-4773.

- Ruusala, T. & Kurland, C. G. 1984, "Streptomycin preferentially perturbs ribosomal proofreading", *Mol.Gen.Genet.*, vol. 198, no. 1, pp. 100-104.
- Ryffel, C., Strassle, A., Kayser, F. H., & Berger-Bachi, B. 1994, "Mechanisms of heteroresistance in methicillin-resistant *Staphylococcus aureus*", *Antimicrobial Agents and Chemotherapy*, vol. 38, no. 4, pp. 724-728.
- SaiSree, L., Reddy, M., & Gowrishankar, J. 2000, "Ion incompatibility associated with mutations causing SOS induction: null *uvrD* alleles induce an SOS response in *Escherichia coli*", *The Journal of Bacteriology*, vol. 182, no. 11, pp. 3151-3157.
- Salazar, L., Fsihi, H., de Rossi, E., Riccardi, G., Rios, C., Cole, S. T., & Takiff, H. E. 1996, "Organization of the origins of replication of the chromosomes of *Mycobacterium smegmatis*, *Mycobacterium leprae* and *Mycobacterium tuberculosis* and isolation of a functional origin from *M. smegmatis*", *Mol.Microbiol.*, vol. 20, no. 2, pp. 283-293.
- Salfinger, M., Crowle, A. J., & Reller, L. B. 1990, "Pyrazinamide and pyrazinoic acid activity against tubercle bacilli in cultured human macrophages and in the BACTEC system", *J Infect.Dis.*, vol. 162, no. 1, pp. 201-207.
- Sancar, A. 1994, "Mechanisms of DNA excision repair", *Science*, vol. 266, no. 5193, pp. 1954-1956.
- Sander, P., De Rossi, E., Boddingtonhaus, B., Cantoni, R., Branzoni, M., Bottger, E. C., Takiff, H., Rodriguez, R., Lopez, G., & Riccardi, G. 2000, "Contribution of the multidrug efflux pump LfrA to innate mycobacterial drug resistance", *FEMS Microbiology Letters*, vol. 193, no. 1, pp. 19-23.
- Sarkar, S. 1991, "Haldane's Solution of the Luria-Delbrück Distribution", *Genetics*, vol. 127, pp. 257-261.
- Sarkar, S., Ma, W. T., & Sandri, G. H. 1992, "On fluctuation analysis: a new, simple and efficient method for computing the expected number of mutants", *Genetica*, vol. 85, no. 2, pp. 173-179.

- Saunders, B. M. & Cooper, A. M. 2000, "Restraining mycobacteria: Role of granulomas in mycobacterial infections", *Immunology and Cell Biology*, vol. 78, no. 4, pp. 334-341.
- Schaaper, R. M. & Dunn, R. L. 1991, "Spontaneous Mutation in the *Escherichia coli lacI* Gene", *Genetics*, vol. 129, no. 2, pp. 317-326.
- Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efron, B., Butcher, P. D., Nathan, C., & Schoolnik, G. K. 2003, "Transcriptional Adaptation of *Mycobacterium tuberculosis* within Macrophages: Insights into the Phagosomal Environment", *The Journal of Experimental Medicine*, vol. 198, no. 5, pp. 693-704.
- Schatz, A. & Waksman, S. 1944, "Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria.", *Proc.Soc.Exp.Biol.Med.*, vol. 57, pp. 244-248.
- Schnarr, M., Oertel-Buchheit, P., Kazmaier, M., & Granger-Schnarr, M. 1991, "DNA binding properties of the LexA repressor", *Biochimie*, vol. 73, no. 4, pp. 423-431.
- Schrag, S. J. & Perrot, V. 1996, "Reducing antibiotic resistance", *Nature*, vol. 381, no. 6578, pp. 120-121.
- Schrag, S. J., Perrot, V., & Levin, B. R. 1997, "Adaptation to the fitness costs of antibiotic resistance in *Escherichia coli*", *Proc.R.Soc.Lond B Biol.Sci.*, vol. 264, no. 1386, pp. 1287-1291.
- Scorpio, A., Lindholm-Levy, P., Heifets, L., Gilman, R., Siddiqi, S., Cynamon, M., & Zhang, Y. 1997, "Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*", *Antimicrobial Agents and Chemotherapy*, vol. 41, no. 3, pp. 540-543.
- Scorpio, A. & Zhang, Y. 1996, "Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus", *Nat.Med.*, vol. 2, no. 6, pp. 662-667.

- Seeberg, E., Eide, L., & Bjoras, M. 1995, "The base excision repair pathway", *Trends Biochem.Sci.*, vol. 20, no. 10, pp. 391-397.
- Selkon, J. B. 1969, "Atypical mycobacteria: a review", *Tubercle*, vol. 50, pp. 70-78.
- Selwyn, P. A., Hartel, D., Lewis, V. A., Schoenbaum, E. E., Vermund, S. H., Klein, R. S., Walker, A. T., & Friedland, G. H. 1989, "A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection", *N.Engl.J Med.*, vol. 320, no. 9, pp. 545-550.
- Shaila, M. S., Gopinathan, K. P., & Ramakrishnan, T. 1973, "Protein synthesis in *Mycobacterium tuberculosis* H37Rv and the effect of streptomycin in streptomycin-susceptible and -resistant strains", *Antimicrobial Agents and Chemotherapy*, vol. 4, no. 3, pp. 205-213.
- Shaw, K. J., Miller, N., Liu, X., Lerner, D., Wan, J., Bittner, A., & Morrow, B. J. 2003, "Comparison of the changes in global gene expression of *Escherichia coli* induced by four bactericidal agents", *J.Mol.Microbiol.Biotechnol.*, vol. 5, no. 2, pp. 105-122.
- Sherman, D. R., Mdluli, K., Hickey, M. J., Arain, T. M., Morris, S. L., Barry, C. E., III, & Stover, C. K. 1996, "Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*", *Science*, vol. 272, no. 5268, pp. 1641-1643.
- Sherman, D. R., Sabo, P. J., Hickey, M. J., Arain, T. M., Mahairas, G. G., Yuan, Y., Barry, C. E., III, & Stover, C. K. 1995, "Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria", *Proc.Natl.Acad.Sci.U.S.A*, vol. 92, no. 14, pp. 6625-6629.
- Shi, L., Sohaskey, C. D., Kana, B. D., Dawes, S., North, R. J., Mizrahi, V., & Gennaro, M. L. 2005, "Changes in energy metabolism of *Mycobacterium tuberculosis* in mouse lung and under *in vitro* conditions affecting aerobic respiration", *Proceedings of the National Academy of Sciences*, vol. 102, no. 43, pp. 15629-15634.

Shibutani, S., Bodepudi, V., Johnson, F., & Grollman, A. P. 1993, "Translesional synthesis on DNA templates containing 8-oxo-7,8-dihydrodeoxyadenosine", *Biochemistry*, vol. 32, no. 17, pp. 4615-4621.

Shibutani, S., Takeshita, M., & Grollman, A. P. 1991, "Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG", *Nature*, vol. 349, no. 6308, pp. 431-434.

Shilova, M. V. & Dye, C. 2001, "The resurgence of tuberculosis in Russia", *Philos.Trans.R.Soc.Lond B Biol.Sci.*, vol. 356, no. 1411, pp. 1069-1075.

Siddiqi, N., Das, R., Pathak, N., Banerjee, S., Ahmed, N., Katoch, V. M., & Hasnain, S. E. 2004, "*Mycobacterium tuberculosis* Isolate with a distinct genomic identity overexpresses a tap-like efflux pump", *Infection*, vol. 32, no. 2, pp. 109-111.

Siddiqi, N., Shamim, M., Hussain, S., Choudhary, R. K., Ahmed, N., Prachee, Banerjee, S., Savithri, G. R., Alam, M., Pathak, N., Amin, A., Hanief, M., Katoch, V. M., Sharma, S. K., & Hasnain, S. E. 2002, "Molecular characterization of multidrug-resistant isolates of *Mycobacterium tuberculosis* from patients in North India", *Antimicrobial Agents and Chemotherapy*, vol. 46, no. 2, pp. 443-450.

Siefert, H. M., Domdey-Bette, A., Henninger, K., Hucke, F., Kohlsdorfer, C., & Stass, H. H. 1999, "Pharmacokinetics of the 8-methoxyquinolone, moxifloxacin: a comparison in humans and other mammalian species", *Journal of Antimicrobial Chemotherapy*, vol. 43 Suppl B, pp. 69-76.

Singapore Tuberculosis Service/British Medical Research Council 1985, "Clinical trial of three 6-month regimens of chemotherapy given intermittently in the continuation phase in the treatment of pulmonary tuberculosis.", *Am.Rev.Respir.Dis.*, vol. 132, no. 2, pp. 374-378.

Slater, S. C., Lifshits, M. R., O'Donnell, M., & Maurer, R. 1994, "*holE*, the gene coding for the theta subunit of DNA polymerase III of *Escherichia coli*: characterization of a

- holE* mutant and comparison with a *dnaQ* (epsilon-subunit) mutant", *The Journal of Bacteriology*, vol. 176, no. 3, pp. 815-821.
- Smith, G. R. 1988, "Homologous recombination in procaryotes", *Microbiol.Rev.*, vol. 52, no. 1, pp. 1-28.
- Smith, P. G. & Moss, A. R. 1994, "Epidemiology of Tuberculosis," in *Tuberculosis: Pathogenesis, Protection, and Control*, B. R. Bloom, ed., American Society for Microbiology, Washington D.C., pp. 47-59.
- Snider, D. E., Graczyk, J., Bek, E., & Rogowski, J. 1984, "Supervised six-months treatment of newly diagnosed pulmonary tuberculosis using isoniazid, rifampin, and pyrazinamide with and without streptomycin", *Am.Rev.Respir.Dis.*, vol. 130, no. 6, pp. 1091-1094.
- Soini, H. & Musser, J. M. 2001, "Molecular Diagnosis of Mycobacteria", *Clinical Chemistry*, vol. 47, no. 5, pp. 809-814.
- Somoskovi, A., Parsons, L. M., & Salfinger, M. 2001, "The molecular basis of resistance to isoniazid, rifampin and pyrazinamide in *Mycobacterium tuberculosis*", *Respiratory Research*, vol. 2, no. 3, pp. 164-168.
- Spargo, C. A., Haaland, P. D., Jurgensen, S. R., Shank, D. D., & Walker, G. T. 1993, "Chemiluminescent detection of strand displacement amplified DNA from species comprising the *Mycobacterium tuberculosis* complex", *Molecular and Cellular Probes*, vol. 7, no. 5, pp. 395-404.
- Springer, B., Master, S., Sander, P., Zahrt, T., McFalone, M., Song, J., Papavinasasundaram, K. G., Colston, M. J., Boettger, E., & Deretic, V. 2001, "Silencing of Oxidative Stress Response in *Mycobacterium tuberculosis*: Expression Patterns of *ahpC* in Virulent and Avirulent Strains and Effect of *ahpC* Inactivation", *Infection and Immunity*, vol. 69, no. 10, pp. 5967-5973.



- Springer, B., Sander, P., Sedlacek, L., Hardt, W. D., Mizrahi, V., Schar, P., & Bottger, E. C. 2004, "Lack of mismatch correction facilitates genome evolution in mycobacteria", *Molecular Microbiology*, vol. 53, no. 6, pp. 1601-1609.
- Sreevatsan, S., Pan, X., Stockbauer, K. E., Connell, N. D., Kreiswirth, B. N., Whittam, T. S., & Musser, J. M. 1997a, "Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination.", *Proceedings of the National Academy of Sciences*, vol. 94, pp. 9869-9874.
- Sreevatsan, S., Pan, X., Zhang, Y., Deretic, V., & Musser, J. M. 1997b, "Analysis of the *oxyR-ahpC* region in isoniazid-resistant and - susceptible *Mycobacterium tuberculosis* complex organisms recovered from diseased humans and animals in diverse localities", *Antimicrobial Agents and Chemotherapy*, vol. 41, no. 3, pp. 600-606.
- Sreevatsan, S., Stockbauer, K. E., Pan, X., Kreiswirth, B. N., Moghazeh, S. L., Jacobs, W. R., Jr., Telenti, A., & Musser, J. M. 1997c, "Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of *embB* mutations", *Antimicrobial Agents and Chemotherapy*, vol. 41, no. 8, pp. 1677-1681.
- Stanford, J. L. 1987, "Much's granules revisited", *Tubercle.*, vol. 68, no. 4, pp. 241-242.
- Stewart, G. R., Robertson, B. D., & Young, D. B. 2004, "Analysis of the function of mycobacterial DnaJ proteins by overexpression and microarray profiling", *Tuberculosis*, vol. 84, pp. 180-187.
- Stewart, G. R., Wernisch, L., Stabler, R., Mangan, J. A., Hinds, J., Laing, K. G., Young, D. B., & Butcher, P. D. 2002, "Dissection of the heat-shock response in *Mycobacterium tuberculosis* using mutants and microarrays", *Microbiology*, vol. 148, no. 10, pp. 3129-3138.
- Stoeckle, M. Y., Guan, L., Riegler, N., Weitzman, I., Kreiswirth, B., Kornblum, J., Laraque, F., & Riley, L. W. 1993, "Catalase-peroxidase gene sequences in isoniazid-

- sensitive and -resistant strains of *Mycobacterium tuberculosis* from New York City", *J.Infect.Dis.*, vol. 168, no. 4, pp. 1063-1065.
- Stone, R. 2000, "SOCIAL SCIENCE: Stress: The Invisible Hand in Eastern Europe's Death Rates", *Science*, vol. 288, no. 5472, pp. 1732-1733.
- Suarez, P. G., Floyd, K., Portocarrero, J., Alarcon, E., Rapiti, E., Ramos, G., Bonilla, C., Sabogal, I., Aranda, I., Dye, C., Raviglione, M., & Espinal, M. A. 2002, "Feasibility and cost-effectiveness of standardised second-line drug treatment for chronic tuberculosis patients: a national cohort study in Peru", *Lancet*, vol. 359, no. 9322, pp. 1980-1989.
- Suarez, P. G., Watt, C. J., Alarcon, E., Portocarrero, J., Zavala, D., Canales, R., Luelmo, F., Espinal, M. A., & Dye, C. 2001, "The dynamics of tuberculosis in response to 10 years of intensive control effort in Peru", *J Infect.Dis.*, vol. 184, no. 4, pp. 473-478.
- Sullivan, E. A., Kreiswirth, B. N., Palumbo, L., Kapur, V., Musser, J. M., Ebrahimzadeh, A., & Frieden, T. R. 1995, "Emergence of fluoroquinolone-resistant tuberculosis in New York City", *Lancet*, vol. 345, no. 8958, pp. 1148-1150.
- Supply, P., Mazars, E., Lesjean, S., Vincent, V., Gicquel, B., & Locht, C. 2000, "Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome", *Molecular Microbiology*, vol. 36, no. 3, pp. 762-771.
- Sutherland, I. 1968, *The 10 year incidence of clinical tuberculosis following conversion in 2550 individuals aged 14 to 19 years. Tuberculosis Surveillance Research Unit Progress Report.*, KNCV, The Hague.
- Suzuki, K., Miyaki, M., Ono, T., Mori, H., Moriya, H., & Kato, T. 1983, "UV-induced imbalance of the deoxyribonucleoside triphosphate pool in *E. coli*", *Mutat.Res.*, vol. 122, no. 3-4, pp. 293-298.
- Taddei, F., Matic, I., & Radman, M. 1995, "cAMP-dependent SOS induction and mutagenesis in resting bacterial populations", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 92, no. 25, pp. 11736-11740.

- Tahaoglu, K., Törün, T., Sevim, T., Ataç, G., Kir, A., Karasulu, L., Özmen, I., & Kapakli, N. 2001, "The treatment of Multi-Drug Resistant Tuberculosis in Turkey.", *The New England Journal of Medicine*, vol. 342, no. 3, pp. 170-174.
- Takayama, K., Armstrong, E. L., Kunugi, K. A., & Kilburn, J. O. 1979, "Inhibition by ethambutol of mycolic acid transfer into the cell wall of *Mycobacterium smegmatis*", *Antimicrobial Agents and Chemotherapy*, vol. 16, no. 2, pp. 240-242.
- Takayama, K., Wang, L., & David, H. L. 1972, "Effect of isoniazid on the *in vivo* mycolic acid synthesis, cell growth, and viability of *Mycobacterium tuberculosis*", *Antimicrobial Agents and Chemotherapy*, vol. 2, no. 1, pp. 29-35.
- Takiff, H. E., Cimino, M., Musso, M. C., Weisbrod, T., Martinez, R., Delgado, M. B., Salazar, L., Bloom, B. R., & Jacobs, W. R., Jr. 1996, "Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in *Mycobacterium smegmatis*", *Proceedings of the National Academy of Sciences*, vol. 93, no. 1, pp. 362-366.
- Takiff, H. E., Salazar, L., Guerrero, C., Philipp, W., Huang, W. M., Kreiswirth, B., Cole, S. T., Jacobs, W. R., Jr., & Telenti, A. 1994, "Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations", *Antimicrobial Agents and Chemotherapy*, vol. 38, no. 4, pp. 773-780.
- Talaat, A. M., Lyons, R., Howard, S. T., & Johnston, S. A. 2004, "The temporal expression profile of *Mycobacterium tuberculosis* infection in mice", *Proc.Natl.Acad.Sci.U.S.A*, vol. 101, no. 13, pp. 4602-4607.
- Taniguchi, H., Aramaki, H., Nikaido, Y., Mizuguchi, Y., Nakamura, M., Koga, T., & Yoshida, S. 1996, "Rifampicin resistance and mutation of the *rpoB* gene in *Mycobacterium tuberculosis*", *FEMS Microbiology Letters*, vol. 144, no. 1, pp. 103-108.
- Tartaglia, L. A., Storz, G., & Ames, B. N. 1989, "Identification and molecular analysis of *oxyR*-regulated promoters important for the bacterial adaptation to oxidative stress", *J.Mol.Biol.*, vol. 210, no. 4, pp. 709-719.

- Teixeira, L., Perkins, M. D., Johnson, J. L., Keller, R., Palaci, M., do, V. D., V, Canedo Rocha, L. M., Debanne, S., Talbot, E., & Dietze, R. 2001, "Infection and disease among household contacts of patients with multidrug-resistant tuberculosis", *Int.J.Tuberc.Lung Dis.*, vol. 5, no. 4, pp. 321-328.
- Telenti, A., Imboden, P., Marchesi, F., Lowrie, D., Cole, S., Colston, M. J., Matter, L., Schopfer, K., & Bodmer, T. 1993, "Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*", *Lancet*, vol. 341, no. 8846, pp. 647-650.
- Telenti, A., Philipp, W. J., Sreevatsan, S., Bernasconi, C., Stockbauer, K. E., Wieles, B., Musser, J. M., & Jacobs, W. R., Jr. 1997, "The emb operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol", *Nat.Med.*, vol. 3, no. 5, pp. 567-570.
- Temesgen, Z., Satoh, K., Uhl, J. R., Kline, B. C., & Cockerill, I. I. I. 1997, "Use of polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis to detect a point mutation in the catalase-peroxidase gene (*katG*) of *Mycobacterium tuberculosis*", *Molecular and Cellular Probes*, vol. 11, no. 1, pp. 59-63.
- Textor, S., Wendisch, V. F., De Graaf, A. A., Muller, U., Linder, M. I., Linder, D., & Buckel, W. 1997, "Propionate oxidation in *E. coli* : evidence for operation of a methylcitrate cycle in bacteria", *Arch.Microbiol.*, vol. 168, pp. 428-436.
- Thierry, D., Brisson-Noel, A., Vincent-Levy-Frebault, V., Nguyen, S., Guesdon, J. L., & Gicquel, B. 1990, "Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS6110, and its application in diagnosis", *Journal of Clinical Microbiology*, vol. 28, no. 12, pp. 2668-2673.
- Tippin, B., Pham, P., & Goodman, M. F. 2004, "Error-prone replication for better or worse", *Trends in Microbiology*, vol. 12, no. 6, pp. 288-295.
- Tomioka, H., Sato, K., Akaki, T., Kajitani, H., Kawahara, S., & Sakatani, M. 1999, "Comparative *in vitro* antimicrobial activities of the newly synthesized quinolone HSR-

- 903, sitafloxacin (DU-6859a), gatifloxacin (AM-1155), and levofloxacin against *Mycobacterium tuberculosis* and *Mycobacterium avium* complex", *Antimicrobial Agents and Chemotherapy*, vol. 43, no. 12, pp. 3001-3004.
- Toole, M. J. & Waldman, R. J. 1988, "An analysis of mortality trends among refugee populations in Somalia, Sudan, and Thailand", *Bull. World Health Organ*, vol. 66, no. 2, pp. 237-247.
- Torres, M. J., Criado, A., Ruiz, M., Llanos, A. C., Palomares, J. C., & Aznar, J. 2003, "Improved real-time PCR for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical isolates", *Diagnostic Microbiology and Infectious Disease*, vol. 45, no. 3, pp. 207-212.
- Tortoli, E. 2003, "Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s", *Clinical Microbiology Reviews*, vol. 16, no. 2, pp. 319-354.
- Tortoli, E., Cichero, P., Piersimoni, C., Simonetti, M. T., Gesu, G., & Nista, D. 1999, "Use of BACTEC MGIT 960 for Recovery of Mycobacteria from Clinical Specimens: Multicenter Study", *Journal of Clinical Microbiology*, vol. 37, no. 11, pp. 3578-3582.
- Toungoussova, O. S., Sandven, P., Mariandyshev, A. O., Nizovtseva, N. I., Bjune, G., & Caugant, D. A. 2002, "Spread of Drug-Resistant *Mycobacterium tuberculosis* Strains of the Beijing Genotype in the Archangel Oblast, Russia", *Journal of Clinical Microbiology*, vol. 40, no. 6, pp. 1930-1937.
- Toungoussova, O. S., Caugant, D. A., Sandven, P., Mariandyshev, A. O., & Bjune, G. 2004, "Impact of drug resistance on fitness of *Mycobacterium tuberculosis* strains of the W-Beijing genotype", *FEMS Immunol. Med Microbiol.*, vol. 42, no. 3, pp. 281-290.
- Tracevska, T., Jansone, I., Broka, L., Marga, O., & Baumanis, V. 2002, "Mutations in the *rpoB* and *katG* genes leading to drug resistance in *Mycobacterium tuberculosis* in Latvia", *Journal of Clinical Microbiology*, vol. 40, no. 10, pp. 3789-3792.

Truffot-Pernot, C., Ji, B., & Grosset, J. 1991, "Activities of pefloxacin and ofloxacin against mycobacteria: *in vitro* and mouse experiments", *Tubercle.*, vol. 72, no. 1, pp. 57-64.

Valim, A. R., Rossetti, M. L., Ribeiro, M. O., & Zaha, A. 2000, "Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* isolates from Brazil", *Journal of Clinical Microbiology*, vol. 38, no. 8, pp. 3119-3122.

Van Der Zwet, W. C., Debets-Ossenkopp, Y. J., Reinders, E., Kapi, M., Savelkoul, P. H., Van Elburg, R. M., Hiramatsu, K., & Vandenbroucke-Grauls, C. M. 2002, "Nosocomial spread of a *Staphylococcus capitis* strain with heteroresistance to vancomycin in a neonatal intensive care unit", *Journal of Clinical Microbiology*, vol. 40, no. 7, pp. 2520-2525.

van Embden, J. D., Cave, M. D., Crawford, J. T., Dale, J. W., Eisenach, K. D., Gicquel, B., Hermans, P., Martin, C., McAdam, R., & Shinnick, T. M. 1993, "Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology", *J Clin. Microbiol.*, vol. 31, no. 2, pp. 406-409.

Vennesland, K., Ebert, R., & Block, R. 1947, "The demonstration of naturally occurring streptomycin resistant variants in the human strain of tubercle bacillus.", *Science* pp. 476-477.

Victor, T. C., Jordaan, A. M., van Rie, A., van der Spuy, G. D., Richardson, M., van Helden, P. D., & Warren, R. 1999, "Detection of mutations in drug resistance genes of *Mycobacterium tuberculosis* by a dot-blot hybridization strategy", *Tubercle and Lung Disease*, vol. 79, no. 6, pp. 343-348.

von Pirquet, C. 1907, "Frequency of tuberculosis in childhood.", *J.Am.Med.Assoc.*, vol. 52, pp. 675-678.

Voskuil, M. I., Schnappinger, D., Visconti, K. C., Harrell, M. I., Dolganov, G. M., Sherman, D. R., & Schoolnik, G. K. 2003, "Inhibition of Respiration by Nitric Oxide

Induces a *Mycobacterium tuberculosis* Dormancy Program", *The Journal of Experimental Medicine*, vol. 198, no. 5, pp. 705-713.

Vynnycky, E. & Fine, P. E. M. 2000, "Lifetime Risks, Incubation Period, and Serial Interval of Tuberculosis", *American Journal of Epidemiology*, vol. 152, no. 3, pp. 247-263.

Waddell, S. J., Stabler, R. A., Laing, K., Kremer, L., Reynolds, R. C., & Besra, G. S. 2004, "The use of microarray analysis to determine the gene expression profiles of *Mycobacterium tuberculosis* in response to anti-bacterial compounds", *Tuberculosis*, vol. 84, no. 3-4, pp. 263-274.

Wagner, J. R., Hu, C. C., & Ames, B. N. 1992, "Endogenous oxidative damage of deoxycytidine in DNA", *Proceedings of the National Academy of Sciences of the USA*, vol. 89, no. 8, pp. 3380-3384.

Walberg, P., McKee, M., Shkolnikov, V., Chenet, L., & Leon, D. A. 1998, "Economic change, crime, and mortality crisis in Russia: regional analysis", *BMJ*, vol. 317, no. 7154, pp. 312-318.

Walker, G. C. 1984, "Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*", *Microbiol.Rev.*, vol. 48, no. 1, pp. 60-93.

Walker, G. C. 1995, "SOS-regulated proteins in translesion DNA synthesis and mutagenesis", *Trends Biochem.Sci.*, vol. 20, no. 10, pp. 416-420.

Walley, J. D., Khan, M. A., Newell, J. N., & Khan, M. H. 2001, "Effectiveness of the direct observation component of DOTS for tuberculosis: a randomised controlled trial in Pakistan", *The Lancet*, vol. 357, no. 9257, pp. 664-669.

Wallis, R. S., Patil, S., Cheon, S. H., Edmonds, K., Phillips, M., Perkins, M. D., Joloba, M., Namale, A., Johnson, J. L., Teixeira, L., Dietze, R., Siddiqi, S., Mugerwa, R. D.,

- Eisenach, K., & Ellner, J. J. 1999, "Drug Tolerance in *Mycobacterium tuberculosis*", *Antimicrobial Agents and Chemotherapy*, vol. 43, no. 11, pp. 2600-2606.
- Wang, J. C. 1985, "DNA Topoisomerases", *Annual Review of Biochemistry*, vol. 54, no. 1, pp. 665-697.
- Watterson, S. A. & Drobniewski, F. A. 2000, "Modern laboratory diagnosis of mycobacterial infections", *Journal of Clinical Pathology*, vol. 53, no. 10, pp. 727-732.
- Watterson, S. A., Wilson, S. M., Yates, M. D., & Drobniewski, F. A. 1998, "Comparison of three molecular assays for rapid detection of rifampin resistance in *Mycobacterium tuberculosis*", *Journal of Clinical Microbiology*, vol. 36, no. 7, pp. 1969-1973.
- Wayne, L. G. & Sohaskey, C. D. 2001, "Nonreplicating persistence of *Mycobacterium tuberculosis*", *Annu.Rev.Microbiol.*, vol. 55, pp. 139-163.
- Weigle, J. J. 1953, "Induction of mutation in a bacterial virus", *PNAS*, vol. 39, pp. 628-636.
- Wells, W. F. 1955, *Airborne contagion and air hygiene*. Harvard University Press, Cambridge.
- Werngren, J. & Hoffner, S. E. 2003, "Drug-susceptible *Mycobacterium tuberculosis* Beijing genotype does not develop mutation-conferred resistance to rifampin at an elevated rate", *J.Clin.Microbiol.*, vol. 41, no. 4, pp. 1520-1524.
- Wernisch, L., Kendall, S. L., Soneji, S., Wietzorrek, A., Parish, T., Hinds, J., Butcher, P. D., & Stoker, N. G. 2003, "Analysis of whole-genome microarray replicates using mixed models", *Bioinformatics*, vol. 19, no. 1, pp. 53-61.
- Wheeler, P. R. & Anderson, P. M. 1996, "Determination of the primary target for isoniazid in mycobacterial mycolic acid biosynthesis with *Mycobacterium aurum* A+", *Biochem.J.*, vol. 318 ( Pt 2), pp. 451-457.



- White, V. L. C. & Moore-Gillon, J. 2000, "Resource implications of patients with multidrug resistant tuberculosis", *Thorax*, vol. 55, no. 11, pp. 962-963.
- Whitney, J. B. & Wainberg, M. A. 2002, "Isoniazid, the frontline of resistance in *Mycobacterium tuberculosis*", *McGill Journal of Medicine*, vol. 6, pp. 114-123.
- WHO 1994, *WHO Tuberculosis Programme: framework for effective tuberculosis control*. WHO/TB.94.179 Geneva, Switzerland..
- WHO 1999, *TB Advocacy, A Practical Guide 1999, WHO Global Tuberculosis Programme*.
- WHO 2000, *World Health Organisation Global Tuberculosis Control WHO/CDS/TB/2000.275*, WHO, Geneva.
- WHO 2001, *A human rights approach to tuberculosis*., World Health Organisation, Geneva.
- WHO 2002a, *Global tuberculosis control - surveillance, planning, financing, WHO Report 2002 WHO/CDS/TB/2002.295*, WHO, Geneva.
- WHO 2002b, *WHO Fact Sheet No. 104, August 2002* 104.
- WHO 2003, *Anti-Tuberculosis Drug Resistance in the World - Report No.2, Prevalence and Trends*, The WHO/IUATLD Global Project on Anti-Tuberculosis Drug Resistance Surveillance, 2.
- WHO 2004, *Global tuberculosis control - surveillance, planning, financing, WHO Report 2004 WHO/HTM/TB/2004.331*, WHO, Geneva.
- WHO 2006, *The Global Plan to Stop TB 2006-2015*.
- WHO & United Nations High Commission for Refugees 1997, *Tuberculosis control in refugee situations: an inter-agency field manual*., WHO, Geneva, 221.

WHO/IUATLD 2000, *Global Project on Anti-tuberculosis Drug Resistance Surveillance. Anti-tuberculosis drug resistance in the world.*, World Health Organisation, Geneva, 2.

Wichelhaus, T. A., Boddingtonhaus, B., Besier, S., Schafer, V., Brade, V., & Ludwig, A. 2002, "Biological Cost of Rifampin Resistance from the Perspective of *Staphylococcus aureus*", *Antimicrobial Agents and Chemotherapy*, vol. 46, no. 11, pp. 3381-3385.

Wilson, M., DeRisi, J., Kristensen, H. H., Imboden, P., Rane, S., Brown, P. O., & Schoolnik, G. K. 1999, "Exploring drug-induced alterations in gene expression in *Mycobacterium tuberculosis* by microarray hybridization", *Proceedings of the National Academy of Sciences*, vol. 96, no. 22, pp. 12833-12838.

Winder, F. G., Collins, P. B., & Whelan, D. 1971, "Effects of ethionamide and isoxyl on mycolic acid synthesis in *Mycobacterium tuberculosis* BCG", *J.Gen.Microbiol.*, vol. 66, no. 3, pp. 379-380.

Wolinsky, E. 1979, "Nontuberculous mycobacteria and associated diseases", *Am.Rev.Respir.Dis.*, vol. 119, no. 1, pp. 107-159.

Wolleswinkel, v., Nagelkerke, N. J., Broekmans, J. F., & Borgdorff, M. W. 2002, "The impact of immigration on the elimination of tuberculosis in The Netherlands: a model based approach", *Int.J Tuberc.Lung Dis.*, vol. 6, no. 2, pp. 130-136.

Wood, M. L., Esteve, A., Morningstar, M. L., Kuziemko, G. M., & Essigmann, J. M. 1992, "Genetic effects of oxidative DNA damage: comparative mutagenesis of 7,8-dihydro-8-oxoguanine and 7,8-dihydro-8-oxoadenine in *Escherichia coli*", *Nucleic Acids Research*, vol. 20, no. 22, pp. 6023-6032.

Woodley, C. L., Kilburn, J. O., David, H. L., & Silcox, V. A. 1972, "Susceptibility of mycobacteria to rifampin", *Antimicrobial Agents and Chemotherapy*, vol. 2, no. 4, pp. 245-249.

Woods, G. L. 2001, "Molecular techniques in mycobacterial detection", *Arch.Pathol Lab Med.*, vol. 125, no. 1, pp. 122-126.

- Wu, S., de Lencastre, H., & Tomasz, A. 1996, "Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing", *The Journal of Bacteriology*, vol. 178, no. 20, pp. 6036-6042.
- Wu, C. H., Tsai-Wu, J. J., Huang, Y. T., Lin, C. Y., Lioua, G. G., & Lee, F. J. 1998, "Identification and subcellular localization of a novel Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis*", *FEBS Lett.*, vol. 439, no. 1-2, pp. 192-196.
- Xu, C., Kreiswirth, B. N., Sreevatsan, S., Musser, J. M., & Drlica, K. 1996, "Fluoroquinolone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*", *J Infect.Dis.*, vol. 174, no. 5, pp. 1127-1130.
- Xu, S., Cooper, A., Sturgill-Koszycki, S., van Heyningen, T., Chatterjee, D., Orme, I., Allen, P., & Russell, D. G. 1994, "Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages", *The Journal of Immunology*, vol. 153, no. 6, pp. 2568-2578.
- Xu, Y., Xie, J., Li, Y., Yue, J., Chen, J., Chunyu, L., & Wang, H. 2003, "Using a cDNA microarray to study cellular gene expression altered by *Mycobacterium tuberculosis*", *Chin Med.J.(Engl.)*, vol. 116, no. 7, pp. 1070-1073.
- Yamori, S., Ichiyama, S., Shimokata, K., & Tsukamura, M. 1992, "Bacteriostatic and bactericidal activity of antituberculosis drugs against *Mycobacterium tuberculosis*, *Mycobacterium avium*-*Mycobacterium intracellulare* complex and *Mycobacterium kansasii* in different growth phases", *Microbiol.Immunol.*, vol. 36, no. 4, pp. 361-368.
- Yang, B., Koga, H., Ohno, H., Ogawa, K., Fukuda, M., Hirakata, Y., Maesaki, S., Tomono, K., Tashiro, T., & Kohno, S. 1998, "Relationship between antimycobacterial activities of rifampicin, rifabutin and KRM-1648 and *rpoB* mutations of *Mycobacterium tuberculosis*", *Journal of Antimicrobial Chemotherapy*, vol. 42, no. 5, pp. 621-628.
- Youatt, J. 1969, "A review of the action of isoniazid", *Am.Rev.Respir.Dis.*, vol. 99, no. 5, pp. 729-749.

- Youmans, G. P. 1979, *Tuberculosis* WB Saunders, Philadelphia.
- Yuen, L. K., Leslie, D., & Coloe, P. J. 1999, "Bacteriological and molecular analysis of rifampin-resistant *Mycobacterium tuberculosis* strains isolated in Australia", *Journal of Clinical Microbiology*, vol. 37, no. 12, pp. 3844-3850.
- Zahrt, T. C., Song, J., Siple, J., & Deretic, V. 2001, "Mycobacterial FurA is a negative regulator of catalase-peroxidase gene *katG*", *Molecular Microbiology*, vol. 39, no. 5, pp. 1174-1185.
- Zambrano, M. M. & Kolter, R. 1996, "GASping for life in stationary phase", *Cell*, vol. 86, pp. 181-184.
- Zhang, Y., Heym, B., Allen, B., Young, D., & Cole, S. 1992, "The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*", *Nature*, vol. 358, no. 6387, pp. 591-593.
- Zhang, Y. & Telenti, A. 2000, "Genetics of drug resistance in *Mycobacterium tuberculosis*," in *Molecular Genetics of Mycobacteria*, G. F. Hatfull & W. R. Jr. Jacobs, eds., ASM Press, Washington DC, pp. 235-254.
- Zhang, Y. & Young, D. 1994, "Molecular genetics of drug resistance in *Mycobacterium tuberculosis*", *Journal of Antimicrobial Chemotherapy*, vol. 34, no. 3, pp. 313-319.
- Zhang, Y. & Young, D. B. 1993, "Molecular mechanisms of isoniazid: a drug at the front line of tuberculosis control", *Trends Microbiol.*, vol. 1, no. 3, pp. 109-113.
- Zhang, Y., Scorpio, A., Nikaido, H., & Sun, Z. 1999, "Role of Acid pH and Deficient Efflux of Pyrazinoic Acid in Unique Susceptibility of *Mycobacterium tuberculosis* to Pyrazinamide", *The Journal of Bacteriology*, vol. 181, no. 7, pp. 2044-2049.
- Zhao, X., Wang, J. Y., Xu, C., Dong, Y., Zhou, J., Domagala, J., & Drlica, K. 1998, "Killing of *Staphylococcus aureus* by C-8-Methoxy Fluoroquinolones", *Antimicrobial Agents and Chemotherapy*, vol. 42, no. 4, pp. 956-958.

- Zhao, X., Xu, C., Domagala, J., & Drlica, K. 1997, "DNA topoisomerase targets of the fluoroquinolones: A strategy for avoiding bacterial resistance", *Proceedings of the National Academy of Sciences*, vol. 94, no. 25, pp. 13991-13996.
- Zhou, J., Dong, Y., Zhao, X., Lee, S., Amin, A., Ramaswamy, S., Domagala, J., Musser, J. M., & Drlica, K. 2000, "Selection of antibiotic-resistant bacterial mutants: allelic diversity among fluoroquinolone-resistant mutations", *J Infect.Dis.*, vol. 182, no. 2, pp. 517-525.
- Zias, J. 1998, "Tuberculosis and the Jews in the ancient Near East: the biocultural interaction.," in *Digging for Pathogens, Ancient Emerging Diseases - Their evolutionary, anthropological and archaeological context*, C. L. Greenblatt, ed., Balaban Publishers, Rehovoth, Israel, pp. 277-295.
- Ziehl, F. 1882, "Zur Färbung des tuberkelbacillus", *Dtsch.Med.Wochenschr.*, vol. 8, p. 451.
- Zimhony, O., Cox, J. S., Welch, J. T., Vilcheze, C., & Jacobs, W. R., Jr. 2000, "Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FASI) of *Mycobacterium tuberculosis*", *Nature Medicine*, vol. 6, no. 9, pp. 1043-1047.























